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TITLE: Enhanced Androgen Signaling With Androgen Receptor Overexpression in the Osteoblast Lineage Controls Skeletal Turnover, Matrix Quality and Bone Architecture

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14. ABSTRACT Androgens have been shown to be important mediators of bone growth and remodeling independent of estrogen. We genetically engineered transgenic mice in which androgen receptor (AR) overexpression is skeletally targeted in two separate models to better understand the role of androgen signaling directly in bone. In the third year, we have completed the analysis of the second line of AR-transgenic mice, AR2.3-transgenic mice (submitted for publication). Enhanced androgen signaling directly in bone results in inhibition of bone formation by differentiated osteoblasts, with a phenotype reflecting low turnover. Comparisons between both models of AR2.3- and AR3.6-transgenic animals suggests that AR transactivation in osteocytes is primarily responsible for mediating the effects of androgen on matrix quality and/or mineralization (inhibitory), while stromal/immature cells mediate effects of androgen on the periosteum and body composition (anabolic). The consequence of androgen action in vivo is compartment-specific; anabolic effects are exhibited exclusively at periosteal surfaces, but in mature osteoblasts androgens inhibit osteogenesis with detrimental effects on matrix quality, bone fragility and whole bone strength (Specific Aim 1). Cell culture models are currently being characterized to determine the effects of androgens on osteoblast-osteoclast communication (Specific aim 2). After a sustained hypogonadal period, gender differences are observed in the response to androgen replacement: both males and females demonstrate improved bone mineral, but females are insensitive to improvements in body composition. AR overexpression in both models worsens the response. These results indicate that direct androgen is generally not anabolic in the skeleton. Differentiation in calvarial cultures from AR2.3-tg mice and gene expression analysis of androgen action in osteocytes (Specific aim 3) are ongoing. Results indicate regulation of genes involved in osteoblast differentiation that may be detrimental.

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Introduction

Androgen deficiency (as a result of aging, hypogonadism, glucocorticoid therapy, or alcoholism), and other behaviors (chronic smoking, malabsorption and bone marrow malignancies) are associated with the development of osteoporosis in men (1). Osteoporosis is also an important and debilitating side effect of androgen deprivation therapy in conjunction with the treatment of prostate cancer (2, 3). At any one time, osteoporosis affects 20 million Americans. Nearly one-quarter of the patients who suffer a hip fracture die within the first year; 50% of patients are unable to walk without assistance; and 33% are totally dependent (4, 5). Of the 1.3 million bone fractures that can be attributed to osteoporosis every year, 150,000 are hip fractures that occur in men with lifetime risk for the development of fracture at nearly 15% (6). In addition, it is also clear that androgens have an important but very much under-appreciated role in women (7). Other health problems may also be affected by androgen action, including atherosclerotic vascular disease, age-related weakness and disability, memory loss, etc. Since osteoporosis is often coupled with a hypogonadal state, developing an understanding of androgen action in the skeleton may provide insight into development of novel therapeutics for the treatment of osteoporosis and metabolic bone disease.

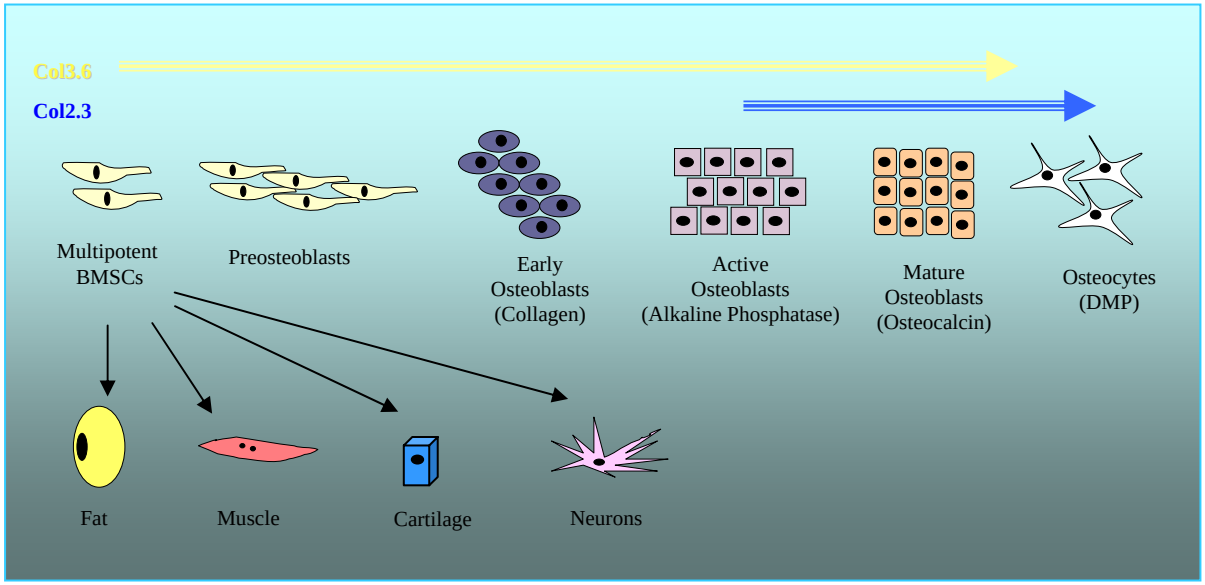
The distinct contribution of androgen to the maintenance of a healthy skeleton remains controversial, since the major androgen metabolite testosterone can serve as the substrate for the production estradiol via aromatase activity. As a consequence, some testosterone action may result from estrogen receptor-dependent activation after conversion to 17-ß estradiol. Overexpression of androgen receptor (AR), combined with the use of non-aromatizable androgens that cannot serve as a substrate for aromatase conversion (e.g. 5α-dihydrotestosterone; DHT), should enhance our understanding of the *specific* role for androgen in bone biology. The goal of this program is to gain a comprehensive understanding of the cascade of molecular and cellular events by which androgen signaling influences skeletal homeostasis.

Our proposed studies have substantial military significance. The stated goals of the Bone Health and Military Readiness program are to advance the understanding of methods to improve bone health of young men and women, to enhance military readiness by reducing the incidence of fracture during physically intensive training, and to reduce the incidence of osteoporosis later in life. As little is known about the direct actions of androgens on osteoblasts, our comprehensive approach using unique animal models of enhanced androgen responsiveness with distinct bone-targeted AR-transgenic families, combined with the novel studies of DHT modulation of osteoblast differentiation and osteoblast-osteoclast signaling, will provide insights into normal bone homeostasis. Understanding the consequences of androgen action in bone is particularly important given increased anabolic steroid abuse. In addition, since bone architecture and bone material properties play important roles in stress fracture, analysis of this model represents a unique opportunity to characterize the consequences of androgen action in both genders on bone microarchitectural quality and the integrity of the skeleton *in vivo*.

Body

In the third year of this grant, we have completed initial characterization of a distinct set of AR-transgenic mice, AR2.3-transgenic families, that were constructed using a smaller collagen promoter fragment to drive AR overexpression in mature osteoblasts (as outlined in Specific Aim 1). Thus, in addition to the AR3.6-transgenic families we have previously created with AR overexpression in stromal cells and throughout the osteoblast lineage including mature osteoblasts, the AR2.3-transgenic mice now also provide models for the characterization of enhanced androgen signaling in distinct skeletal compartments (see Fig. 1). Importantly in both

models, enhanced androgen action occurs only in those cells with elevated levels of AR (skeletally-targeted) as a consequence of enhanced AR signaling, without changes in circulating steroid levels and without systemic androgen administration. Because of distinct and overlapping expression profiles as shown in the schematic below, comparison of the skeletal phenotypes characterized in these two models of enhanced androgen action are postulated to aid in the identification of cells within the osteoblast lineage that are most important in mediating a specific response in bone modeling/remodeling characteristics. For example, phenotypes identified as similar in both AR2.3- transgenic and AR3.6-transgenic suggests that mature osteoblasts/osteocytes are important mediators of the response, since there is overlap in promoter activity in those cell types. In contrast, phenotypes that are more pronounced in AR3.6- transgenic than AR2.3-transgenic suggest that stromal or immature osteoblasts are primary mediators.



Our goal as proposed in Specific Aim 1 is to contrast the skeletal phenotype of AR2.3-transgenic with AR3.6-transgenic animals (with different AR overexpression profiles in the osteoblast lineage), in the adult and in the hypogonadal state in both genders, to identify direct androgen actions *in vivo*. This analysis will allow us to test the hypothesis that distinct profiles of AR overexpression in the osteoblast lineage will result in distinct skeletal phenotypes between AR2.3- transgenic vs. AR3.6-transgenic mice. The progress report is divided into three sections: **1. Studies proposed in Specific aim 1** to compare and contrast the phenotype in AR2.3- transgenic and AR3.6-transgenic lines: a) completion of analysis of the adult AR2.3-transgenic mouse model (manuscript submitted); b) analysis of DHT replacement studies in both a low and high bone turnover situation after gonadectomy in both males and females in both AR2.3- and AR3.6-transgenic mice for evaluation of bone parameters; c) analysis of body composition changes in low and high turnover gonadectomized mice. **2. Studies proposed in Specific aim 2** to determine the importance of AR in regulating osteoclast formation and activation: a) characterization of *ex vivo* cultures derived from AR3.6-transgenic mice. **3. Studies proposed in Specific aim 3** to characterize the role of androgen in regulation osteoblast differentiation: a) microarray analyses of the effects of androgen in osteocytes; and b) characterization of proliferation and differentiation in AR2.3-transgenic calvarial osteoblast (mOB) cultures.

Section 1: Contrast of the skeletal phenotype observed in AR2.3-transgenic with AR3.6-transgenic models.

An important advantage of the both the AR3.6-transgenic and AR2.3-transgenic mouse models are the enhancement of androgen signaling as a consequence of increased AR abundance in likely target (tissues or cells) for androgen *in vivo*, i.e., periosteal cells and the osteoblast lineage compared to mature osteoblasts and osteocytes. These models, characterized by the absence of differences in circulating testosterone or 17ß-estradiol and studied without systemic androgen administration, thus takes advantage of increased sensitivity to androgen in distinct skeletal sites for the analysis of compartment-specific effects of androgen. At the same time, AR overexpression, rather than systemic administration, excludes action at other androgen target tissues *in vivo* including muscle and fat. AR overexpression targeted by the col2.3 promoter was chosen for several reasons: the skeletal expression patterns for this promoter are both well-characterized and bone-selective (see 8, 9); the col2.3 promoter is not active in the periosteum but is strongly expressed in osteocytes and mineralizing nodules (10); the col2.3 promoter is also not active in osteoclasts; and androgens do not inhibit expression from the 2.3 kb promoter fragment (data not shown). Thus, the col2.3 promoter fragment directs expression of the fused transgene in bone, with strong expression still observed at the age of 3 months and even in animals as old as 6 months (10). Reports describing characterization of expression indicated strong expression in cells at osteogenic fronts of parietal bones, but the suture area was negative. In long bones, strong transgene expression was observed in most osteoblasts on endosteal surfaces, and in a large proportion of osteocytes in femurs throughout cortical bone, with no expression seen in periosteal fibroblasts (11). In the trabecular area of metaphyseal bone, strong expression was observed at all developmental stages (10).

1a) *Analysis of the adult AR2.3-transgenic mouse model:*

Confusion exists regarding the *in vivo* action of androgens in bone due to metabolism to estrogen, and because androgen influences many tissues in the body and many months of treatment are required to observe improvement in BMD. The AR2.3-transgenic animal model was created to determine the specific physiologic relevance of androgen action in the mature osteoblast /osteocyte population in bone, through tissue-specific overexpression of AR. This line is distinct from our previously generated transgenic model with AR overexpression in stomal precursors, periosteal fibroblasts and throughout the osteoblast lineage, the AR3.6-transgenic line (12). A transgene cassette (AR2.3) was cloned and AR2.3-transgenic mice were created following standard procedures. Positive founders were identified by PCR genotyping and were bred to wild-type B6D2F1 mice; two AR2.3-transgenic lines (lines 219 and 223) derived from independent founders have been retained. Southern analysis confirmed a single insertion site for the AR2.3-transgene (data not shown). Characterization of expression of the AR2.3-transgene by qRT-PCR analysis in various tissues shows the expected bone targeting, with highest levels in calvaria but ~100-3000 fold lower levels in muscle, skin, heart, intestine, kidney, liver, lung and spleen.

Phenotype in AR2.3-transgenic mice with bone-targeted AR overexpression.

To begin to characterize the phenotype of AR2.3-transgenic mice, we first determined body weight gain and nose-rump length over a 6-month period. At birth, animals were indistinguishable and as the mice aged, AR2.3-transgenic males and females gained length and weight similar to wild-type controls (Fig. 1A, B).

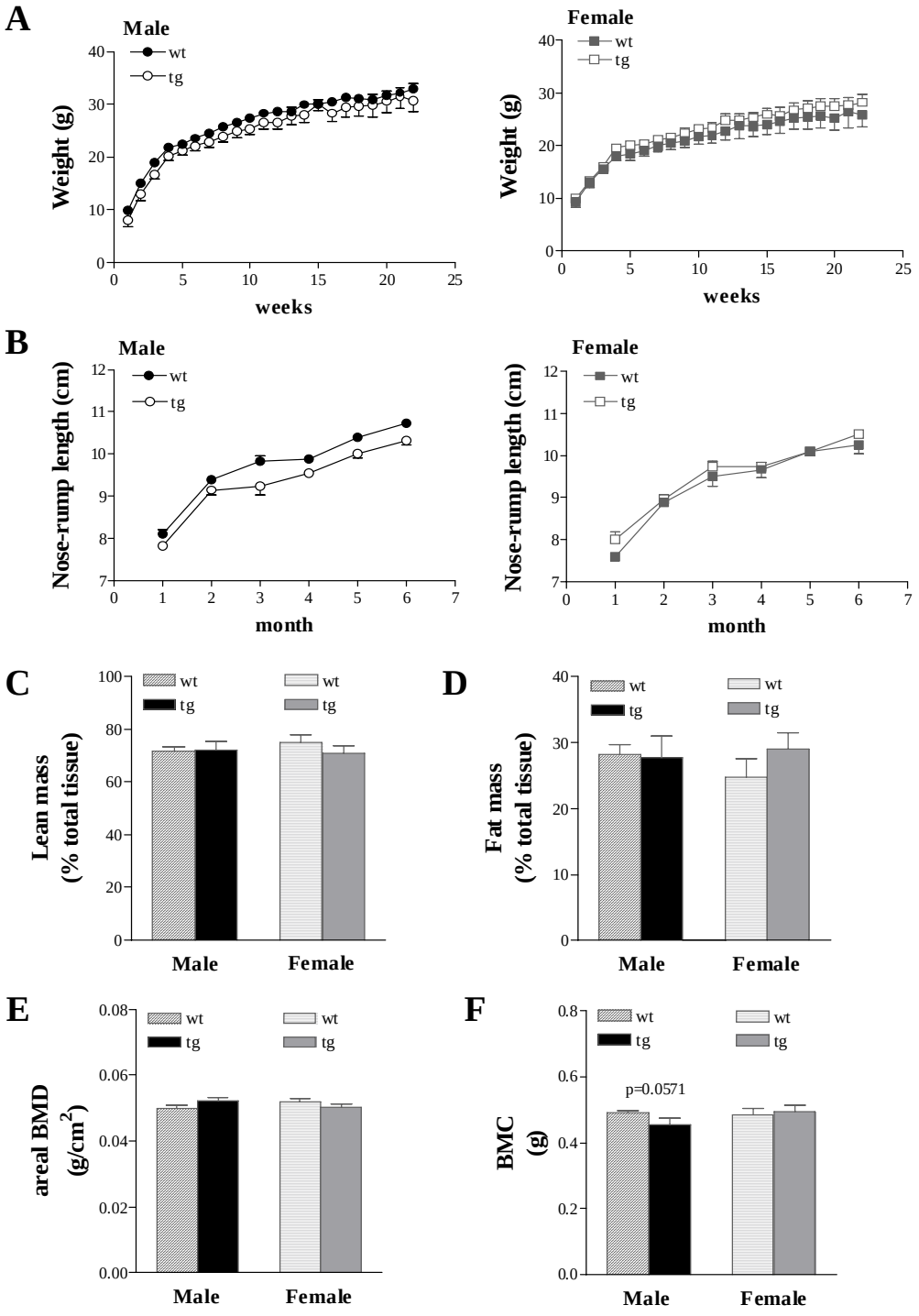


Figure 1. Weight changes and body composition analysis in AR2.3-transgenic mice.
Body weight and nose-rump-length determinations were carried out weekly or monthly, respectively, over six months in both genders in both wild-type (wt) and AR2.3-transgenic (AR2.3-transgenic) mice. **A**. Weight gain in growing male (*left*) and female (*right*) mice. Analysis for the effects of time and genotype by repeated measures two-way ANOVA in males revealed an extremely significant effect of time ($F = 218.36$; $p < 0.0001$) but not genotype, and with no interaction; females were similar ($F = 114.80$; $p < 0.0001$). **B**. Nose-rump length in male (left) and female (right) mice. Analysis by repeated measures in males revealed an extremely significant effect of time ($F = 228.54$; $p < 0.0001$) and an effect of genotype ($F = 15.87$; $p < 0.01$) with no interaction; females only showed an effect of time ($F = 149.48$; $p < 0.0001$). Data is shown as mean ± SEM, n = 4-5. DXA was performed on 6-month-old AR2.3-transgenic and littermate control mice to assess bone mineral, lean mass and fat mass. **C**. Lean mass adjusted for total tissue mass. **D**. Fat mass adjusted for total tissue mass. **E**. Areal BMD (minus head). **F**. BMC. A trend for decreased BMC was noted in male AR2.3-transgenic mice (p=0.0571). Values are expressed as mean ± SEM, n = 4-10.

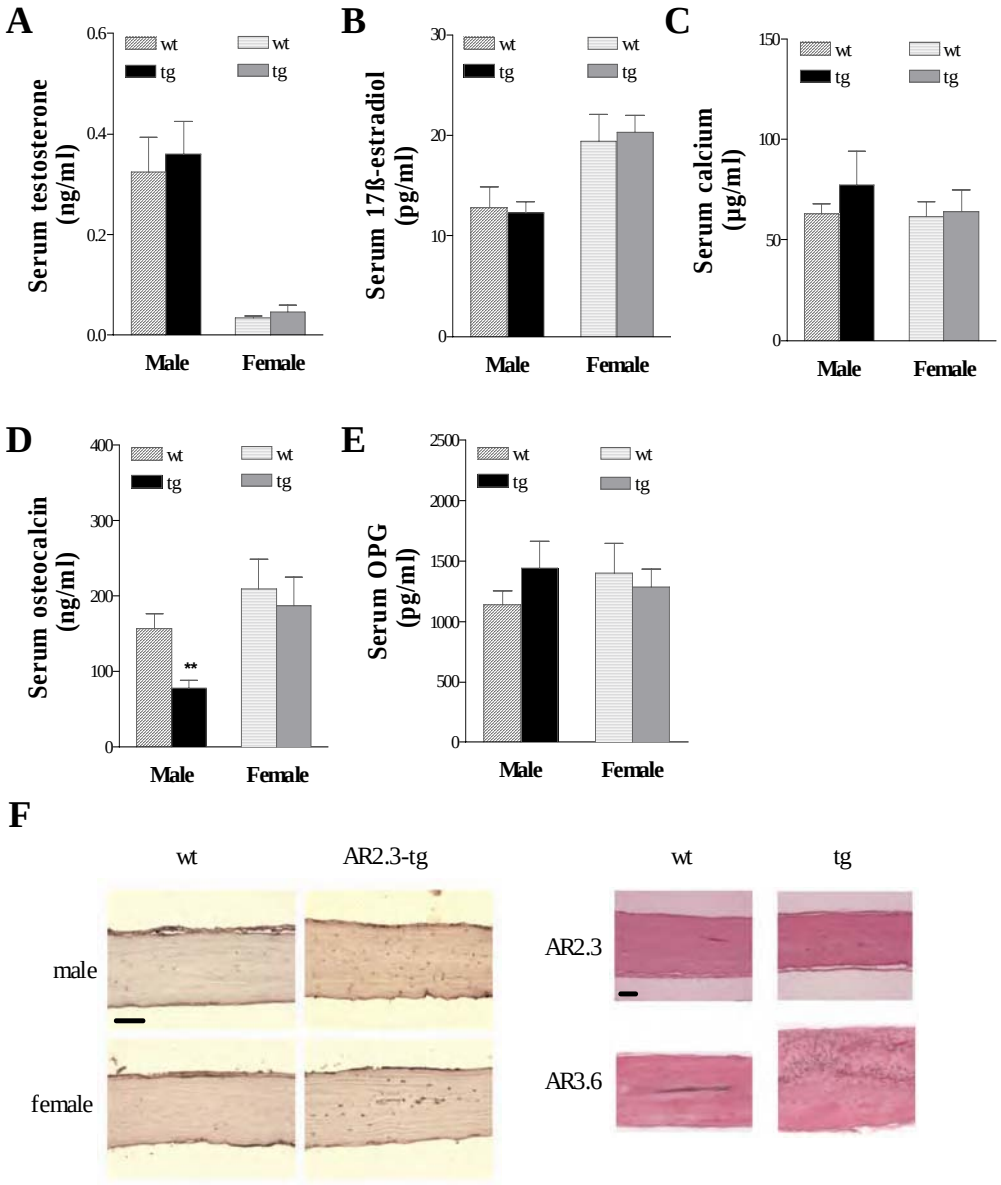


Figure 2. **Phenotypic characterization of serum markers and calvarial thickness in AR2.3-transgenic animals**.
Comparisons were performed between wild-type littermate control (wt) and AR2.3-transgenic (AR2.3-transgenic) animals. Serum from 2-month-old mice was analyzed to determine levels of hormones and markers of calcium metabolism. Assays were performed in duplicate by RIA for 17ß-estradiol or EIA for testosterone, OPG and intact mouse osteocalcin, and for calcium by the colorimetric cresolphthalein-binding method. **A**. Testosterone. **B**. 17ß-estradiol. **C**. Calcium. There were no statistical differences between the genotypes for 17ß-estradiol, testosterone or calcium levels. **D**. Osteocalcin levels were significantly reduced in male AR2.3-transgenic mice. **E.** OPG circulating levels were elevated in males but not in female AR2.3-transgenic mice. Values are expressed as mean ± SEM, n=6-17. **, $p < 0.01$ (*vs*. gender-appropriate wild-type control). **F**. Histological and immunohistochemical analysis of calvaria isolated from 2-month-old mice. Sections were subjected to either H&E staining or immunohistochemical staining after demineralization and paraffin embedding. Representative sections are shown. **Left**. AR abundance was visualized with rabbit polyclonal antisera for male and female mice from wt and AR2.3-transgenic mice. AR is brown and the nucleus is purple after after DAB incubation and counterstaining with hematoxylin. The majority of osteoblasts and osteocytes demonstrated AR immunoreactivity. **Right.** Calvaria from male wild-type, AR2.3-transgenic or AR3.6-transgenic mice were evaluated for increased thickness and periosteal bone formation. Scale bar = 50 µm.

Body composition and bone density were evaluated by DXA at 2 months in male and female AR2.3-transgenic mice and wild-type littermate controls (Fig. 1C). While systemic androgen treatment is known to affect body compositional changes, no difference was noted in either lean mass or fat mass in either males or females (Fig. 1C, D), consistent with skeletal targeting of the AR transgene. In addition, areal BMD was not affected (Fig. 1E), indicating a lack of effect on periosteal surfaces. However, BMC showed a trend for reduction in male transgenic mice (Fig. 1F).

AR2.3-transgenic mice were next evaluated for serum biochemistry and hormone levels at 2 months of age. As expected because of bone-targeting of AR overexpression, serum testosterone and estradiol levels were not significantly different between littermate controls and transgenic animals in either sex (Fig. 2A, B). There were also no significant differences in serum calcium levels between transgenic mice and littermate controls (Fig. 2C). Interestingly, there was a significant ~50% decrease in serum osteocalcin levels in male AR2.3-transgenic animals but not in females ($P < 0.01$, Fig. 2D). Serum OPG, an important inhibitor of osteoclastogenesis (13), was also analyzed. A modest but non-significant increase in serum OPG was observed in AR2.3-transgenic males, again with little effect in transgenic females compared to littermate controls (Fig. 2E).

To further evaluate the phenotype of AR2.3-transgenic mice, we characterized AR protein expression *in vivo* by immunocytochemical analysis in calvarial sections from both sexes. In this analysis, immunostaining represents combined AR levels with detection of both endogenous AR and the product of the AR2.3-transgene. In transgenic mice, the majority of osteoblasts and osteocytes demonstrated AR immunoreactivity (Fig. 2F, left panels), consistent with increased AR2.3-transgene expression. Both male and female transgenic animals demonstrate higher level of AR expression, with no notable difference between the sexes or between independent families (data not shown). Morphological changes were characterized by H&E staining (Fig. 2F right panels). Again, there was no difference between wild-type and AR2.3-transgenic mice of either gender, nor between the independent AR2.3-transgenic families 219 and 223 (data not shown). In contrast to these findings, it is noteworthy that the calvaria from male AR3.6-transgenic mice, with AR overexpression in periosteal fibroblasts, demonstrate substantial calvarial thickening (Fig. 2F right panels).

Enhanced androgen signaling results in altered bone morphology and reduced cortical area in male transgenic mice.

Overall bone morphology and femoral structure were quantified from high resolution μCT images. Measures from the μCT analysis for morphological assessment are described in Fig. 3A. No effect of AR2.3-transgene expression on total cross-sectional area or surface periosteal perimeter was observed in either males or females (Fig. 3B, E). However, marrow cavity area was significantly increased in transgenic males (*i.e.*, reduced infilling; Fig. 3C). Given no compensatory changes in the periosteal layer, this inhibition results in a modest but significant reduction in cortical bone area (Fig. 3D) in male AR2.3-transgenic mice. Thus, enhanced AR signaling in mature osteoblasts has significant inhibitory effects on overall femoral cortical bone area. This morphological difference at the diaphysis was not observed in female transgenic mice. We also evaluated polar moment of inertia and tissue mineral density (TMD) at the mid-diaphysis. There was no significant effect on polar moment of inertia in either male or female AR2.3-transgenic mice (Fig. 3F), but males show a significant reduction in TMD ($P < 0.001$; Fig. 3G), consistent with the reduction in BMC (shown in Fig. 1F).

Reduced endosteal bone formation with bone-targeted AR overexpression.

Because of the changes in cortical bone area observed in the μCT analysis in males, dynamic histomorphometric analysis using fluorescent imaging was carried out at the femoral diaphysis. Fluorochromes were administered (oxytetracycline followed by calcein) to label new mineral deposition. Figure 3H shows patterns of bone formation in images of fluorochrome labeling from femoral cross sections. The AR2.3-transgenic males (upper panel) demonstrate a dramatic lack of labeling at the endosteal surface compared with wild-type controls (lower panel). Consistent with these fluorescent images, quantitative dynamic histomorphometric analysis revealed inhibitory responses at the endosteal surface. Dramatic inhibition (~70%) of both BFR ($P < 0.001$; Fig. 3I) and MAR ($P < 0.001$; Fig. 3J), a measure of osteoblast vigor, were noted at the endosteal surface. Eroded surface was reduced at the periosteal surface ($P < 0.001$; Fig. 3K), and labeled perimeter (L.pm) showed inhibition at the endosteal surface ($P < 0.01$; Fig. 3L) but stimulation at the periosteal surface ($P < 0.01$). The modest increase in periosteal activity does not parallel changes in cortical bone morphology that were observed by μCT analysis in Figure 3E, likely since the labeling is representative of mineralization patterns only for the period of time that the labels are present.

Ability to resist fracture is impaired in male AR2.3-transgenic mice.

To analyze whole bone biomechanical and failure properties, femurs from 2-month-old wild-type and AR2.3-transgenic animals were loaded to failure in 4-point bending at 0.05 mm/s. Although overall geometry of the femur demonstrated no obvious differences between wild-type and transgenic mice (Fig. 4A), failure properties were significantly impaired. Diaphyseal strength as maximum load ($P < 0.05$, Fig. 4B) and stiffness ($P < 0.05$, Fig. 4C) were decreased by about 10% in male AR2.3-transgenic mice (with no change in females), consistent with the decreased cortical bone area in these mice (see Fig. 3C). Male transgenic bones, however, were dramatically impaired in their ability to resist fracture. They were more brittle, with an approximately 40% decrease in post-yield deflection ($P < 0.05$, Fig. 4D), and work-to-failure ($P < 0.05$, Fig. 4E) was reduced by nearly 30% compared to wild-type control bones. Male AR2.3-transgenic mice in this cohort showed no difference in femoral length or weight (Fig. 4F, G).

Among the most striking biomechanical characteristics of the bone from AR2.3-transgenic mice was its ***markedly impaired fracture resistance***. Long bones were significantly more brittle and consequently showed a large decrease in work-to-failure. The inhibition in bone quality appears to be principally determined by changes in the organic matrix of bone, through the ~50% reduction in MAR at the endosteal surface (which reflects osteoblast vigor or work), leading to a detrimental change in the composition of the material properties of the organic matrix and thus a worsening in PYD and work-to-failure. The increased brittleness (decreased post-yield deflection) of cortical bone is not likely due to over-mineralization, since both TMD and BMC were reduced in transgenic males, not increased. Brittleness, and its opposite, ductility, are functional attributes that in bone derive principally from matrix composition and collagen organization rather than bone geometry and mass, which are the major determinants of bone stiffness and strength. Thus, the brittleness observed in these AR transgenic mouse bones points to a defect in bone matrix quality. This in turn suggests a ***defect in osteoblast production of a functionally appropriate bone matrix in the presence of enhanced androgen signaling in mature osteoblasts/osteocytes***. Indeed, our molecular analysis of expression differences from the AR transgenic mice shown below in Fig. 6 demonstrate dramatically reduced collagen and osteocalcin production, consistent with the impaired matrix quality in these mice.

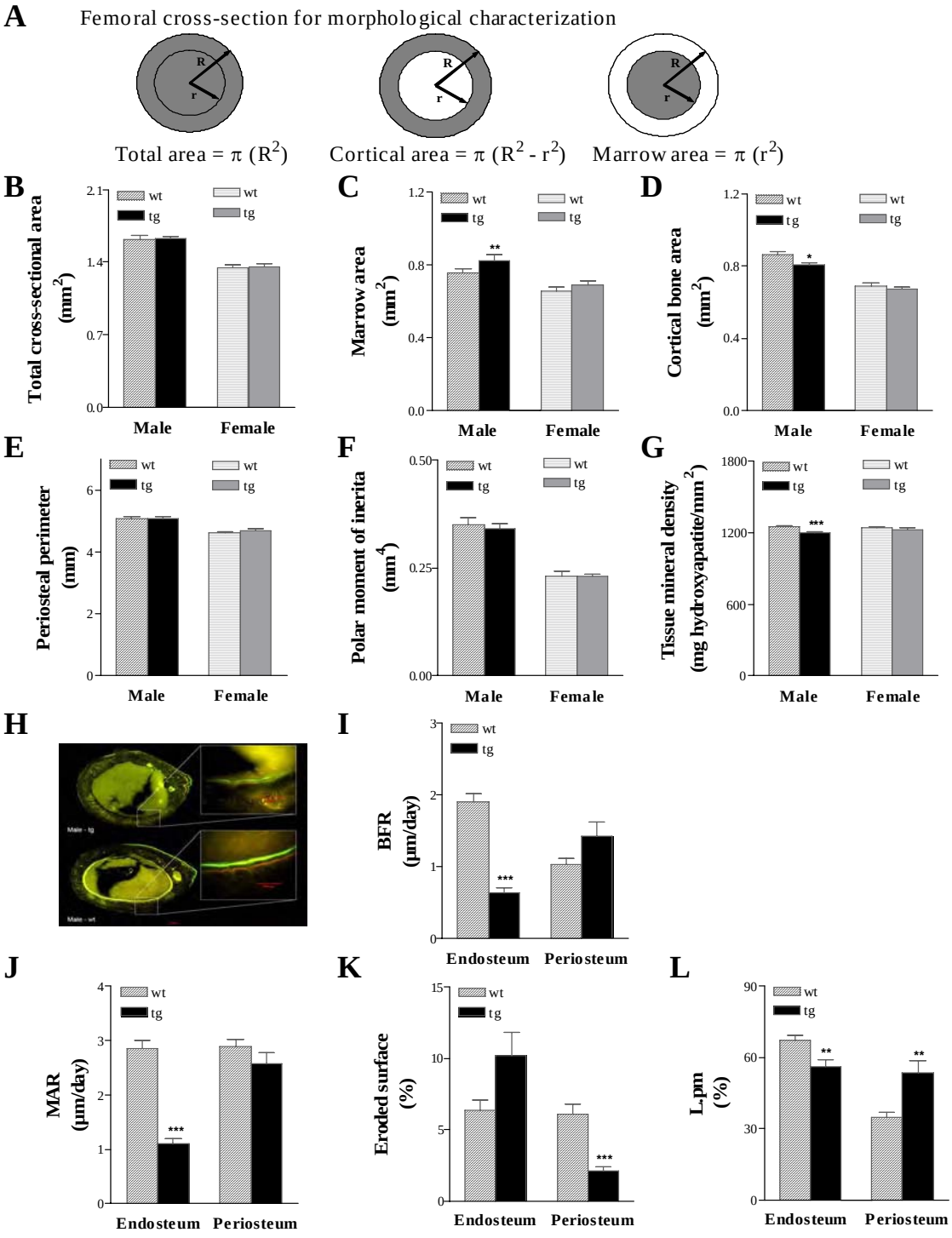


Figure 3. Cortical morphology, structural analysis and bone formation rates in AR2.3-transgenic mice.
Femurs were isolated from 2-month-old male and female wild-type (wt) or AR2.3-transgenic mice (transgenic) and subjected to high resolution µCT imaging at mid-diaphysis. **A.** Parameters for morphological characterization by µCT. **B**. Total cross-sectional area. **C**. Marrow cavity area. **D**. Cortical bone area. **E**. Periosteal perimeter. **F**. Polar moment of inertia. **G**. Tissue mineral density. Values are shown as mean ± SEM, n = 10-21 males; 13-19 females. Differences between genotypes were determined by Student's unpaired *t*-test with Welch's correction. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (*vs*. gender-appropriate wt controls). For dynamic histomorphometric analysis, male femurs were sectioned at the mid-diaphysis; rates were determined at both the endosteal and periosteal surfaces. **H**. Fluorescent images of femur after double-label administration. Representative photomicrographs are shown with higher power insets demonstrating labeling on the endosteal surface. Bands were photographed at comparable anatomic positions for each bone. **I**. Bone formation rate (BFR). **J**. Mineral apposition rate (MAR). **K**. Percent eroded surface. **L**. Percent labeled perimeter (L.pm). Values are shown as mean ± SEM; n = 8-20 males; 10-15 females. Differences between genotypes were determined by Student's unpaired *t*-test with Welch's correction. **, $p < 0.01$; ***, $p < 0.001$ (*vs*. wt controls). Scale bar = 200 µm in figure; scale bar = 100 µm in insets as indicated.

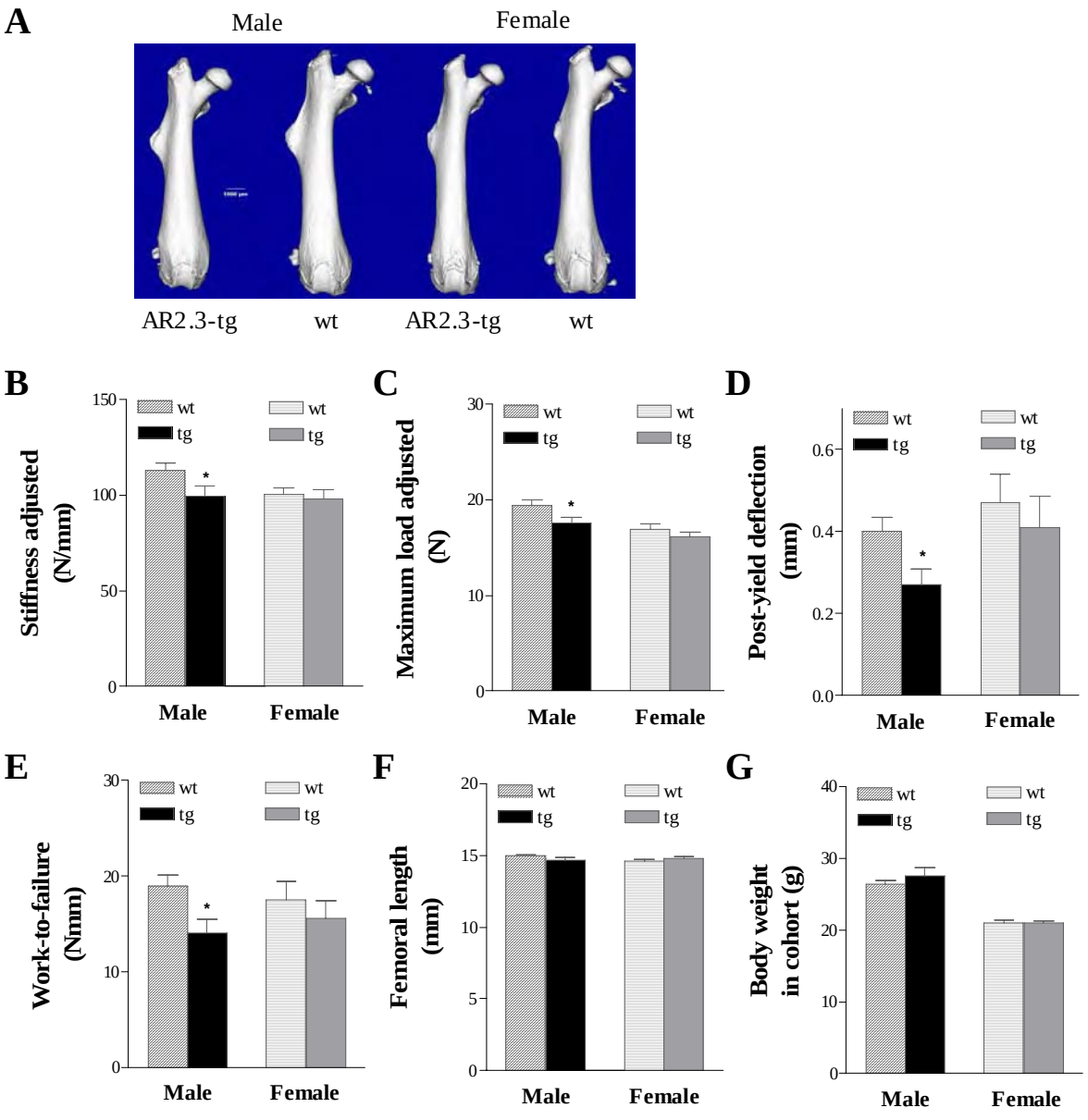


Figure 4. Whole bone strength and failure properties determined from biomechanical analyses.
Femurs from 2-month-old male and female wild-type (wt) and AR2.3-transgenic (transgenic) mice were loaded to failure in 4-point bending analysis. Stiffness, maximum load, and post-yield deflection were calculated from the load-deflection curves. Stiffness and maximum load are adjusted for body weight differences. **A**. Whole bone morphology from µCT imaging. **B**. Stiffness Adjusted. **C**. Maximum Load Adjusted. **D**. Post-yield deflection. **E**. Work-to-failure. **F**. Femoral Length. **G**. Body weight in cohort. Whole bone biomechanical properties are shown as mean ± SEM, n = 10-21 males; 13-19 females. Differences between genotypes were determined by Student's unpaired *t*-test with Welch's correction. *, $p < 0.05$ (*vs*. gender-appropriate wt controls).

AR overexpression leads to increased trabecular bone volume in male transgenic mice.

To determine the consequences of AR overexpression on the trabecular bone compartment, we used µCT analysis. Visualization of trabecular bone in the metaphysis after manual subtraction of the cortical shell shows that trabecular bone volume is increased in male AR2.3-transgenic mice (Fig. 5A). To better characterize trabecular micro-anatomy and architecture, static histomorphometric analysis was performed from images of the metaphyseal trabecular region (Fig. 5B-E). Male AR2.3-transgenic mice showed a ~50% increase in trabecular bone volume as a percent of tissue volume (BV/TV; $P < 0.05$; 5B), consistent with the µCT image. The increase in trabecular bone volume was associated with an ~25% increase in trabecular number (Tb.N; $P < 0.01$; 5C), no effect on trabecular thickness (Tb.Th; 5D), and thus a ~30% decrease in spacing (Tb.Sp; $P < 0.01$; 5E).

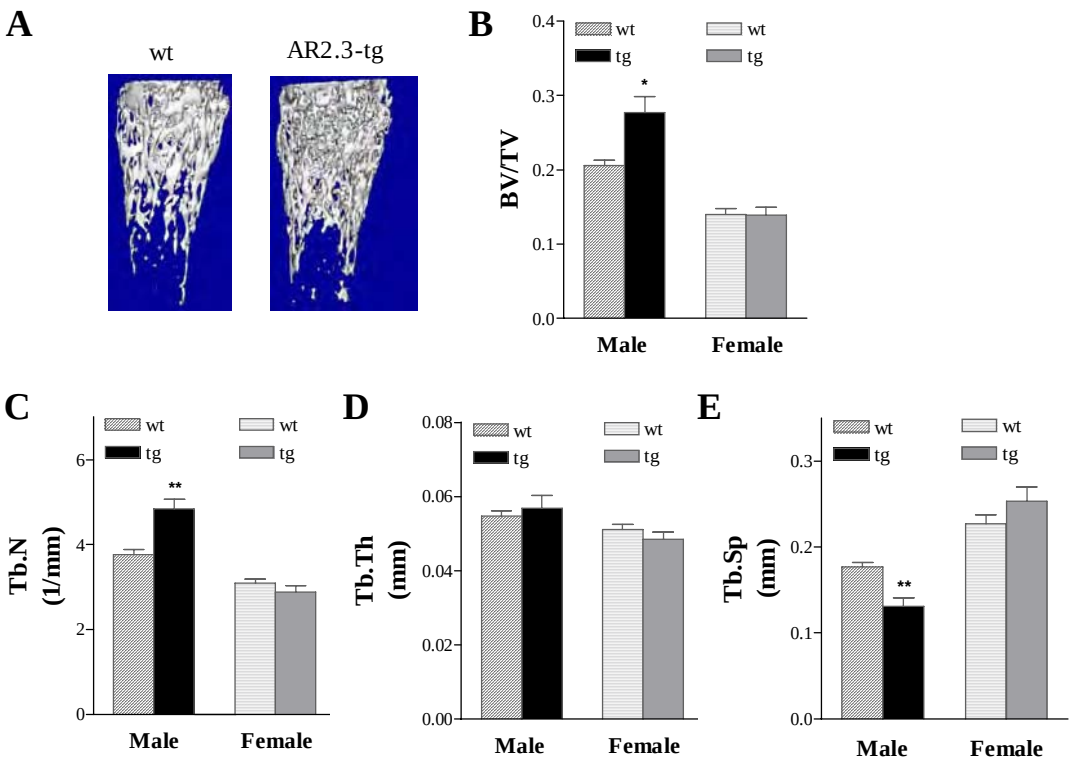


Figure 5. Trabecular morphology and micro-architecture in AR2.3-transgenic mice.
Computer-aided analysis of µCT images was used to derive measures of trabecular bone micro-architecture in the metaphysis of 2-month-old male and female wild-type (wt) or AR2.3-transgenic (transgenic) mice. Measurements included trabecular bone volume as a percent of tissue volume (BV/TV); trabecular number, spacing, and thickness (Tb.N, Tb.Sp, Tb.Th). **A**. Reconstructed images were evaluated for trabecular morphology in the distal metaphysis. **B**. BV/TV. **C**. Tb.N. **D**. Tb.Th. **E**. Tb.Sp. Values are expressed as mean ± SEM, n = 10-21 males; 13-19 females. Differences between genotypes were determined by Student's unpaired *t*-test with Welch's correction. *, $p < 0.05$; **, $p < 0.01$ (*vs*. gender-appropriate wt controls).

Enhanced androgen signaling in mature osteoblasts leads to reduced expression of molecular markers of bone formation and osteoclast activation in cortical bone.
Lastly, we analyzed gene expression in long bone from wild-type and AR2.3-transgenic mice of

both genders. Differences in gene expression in RNA isolated from tibial mid-diaphysis were determined by qRT-PCR analysis and included genes important in both osteoblast and osteoclast activity/development (Fig. 6). Osteoblastic genes evaluated were cyclin D1, osterix, type I collagen (col), and osteocalcin (OC). Levels of osteoblastic genes are listed in an order reflecting their temporal expression patterns during osteoblast differentiation, e.g., osteocalcin is expressed late in osteoblast differentiation. Osteoclastic genes analyzed were OPG, receptor activator of NF-κB ligand (RANKL), tartrate-resistant acid phosphatase (TRAP), cathepsin K (CatK) and calcitonin receptor (CTR). Interestingly, the level of inhibition of expression of osteoblastic genes in mid-diaphyseal tissue from male transgenic mice increased with the differentiation stage, from proliferating osteoblasts to osteocytes, and suggest alteration of the organic matrix. ***The pattern of variation in expression mirrors the pattern of AR overexpression under control of the col2.3 promoter.*** Inhibition of gene expression was also observed in osteoclast genes in male AR-transgenic mice, with the exception of the increase in OPG expression (secreted from osteoblasts). Both the modest increase in OPG as well as the reduction in osteocalcin expression in transgenic males are comparable to the results observed for serum levels of these proteins (see Fig. 2E and 2D, respectively). Consistent with the lack of a bone phenotype in females, there was little difference in expression in female transgenics compared to control mice for any of the osteoblastic or osteoclastic genes analyzed.

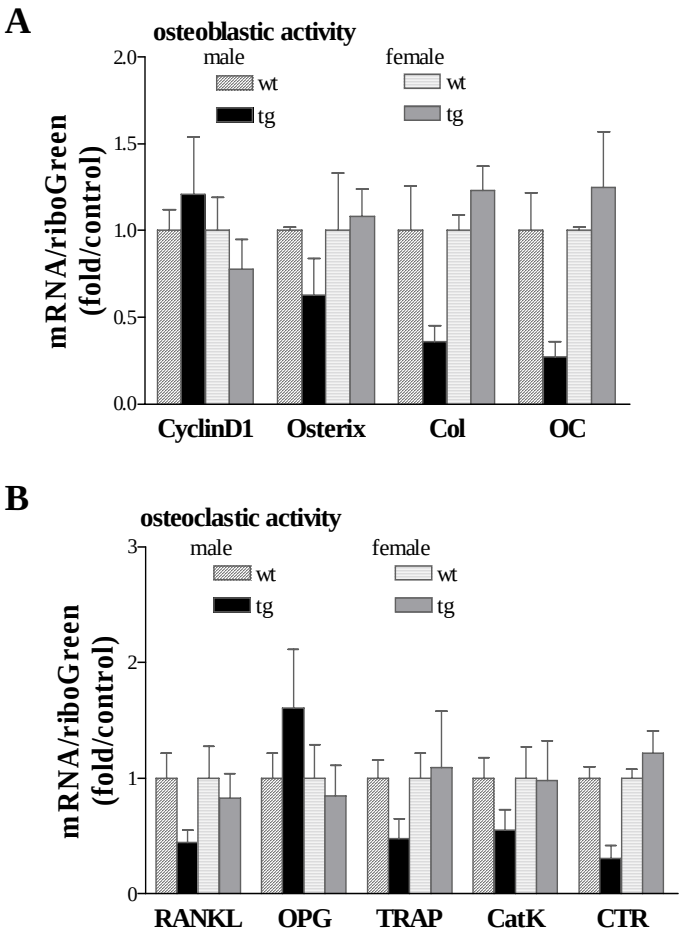


Figure 6. Molecular markers of osteoblasts and osteoclasts in cortical bone by qRT-PCR analysis.
Analysis of steady-state mRNA expression characteristic of osteoblastic or osteoclastic cells was determined by real-time qRT-PCR analysis using tibial RNA isolated from male and female wild-type (wt) or AR2.3-transgenic mice (transgenic). Osteoblast genes involved in bone formation and matrix production examined included cyclin D1, osterix, type I α_1 collagen (Col), and osteocalcin (OC). Genes involved osteoclastic activity and bone resorption were osteoprotegerin (OPG), RANK ligand (RANKL), tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR) and cathepsin K (CatK). **A.** Determination of osteoblast gene expression in males and female mice. **B.** Analysis of osteoclastic gene expression in males and females. n = 4-5 males; 2-8 females. Values are expressed as mean ± SEM.

Comparison between AR2.3-transgenic and AR3.6-transgenic models.

It is instructive to compare the skeletal phenotypes that develop in the two distinct lines that we have generated, the AR2.3-transgenic mice described in these studies and the previously characterized AR3.6-transgenic model (12). In broad terms, the skeletal phenotype characterized in AR2.3-transgenic mice mirrors that described previously for AR3.6-transgenic males, indicating the specificity and reproducibility of the phenotypic consequences of bone-targeted androgen signaling. In common between the two models, we have shown increased trabecular bone volume, reduced formation at endosteal surfaces, reduced bone turnover and compromised biomechanical strength in male transgenic mice. With the exception of enhanced periosteal activity in AR3.6-transgenic males as noted below, neither model exhibits anabolic responses in the cortical bone compartment and instead both show inhibition of bone formation at the endosteal surface and compromised biomechanical properties. By comparing and contrasting the two AR-transgenic models, we propose that the commonalities in the bone phenotype between AR2.3-transgenic and AR3.6-transgenic mice arise from AR overexpression in mature osteoblasts and osteocytes, since both promoters are active in these cells. Thus, the increased trabecular bone volume, reduced bone turnover, reduced formation and decreased osteoblast vigor at endosteal surfaces, and compromised biomechanical strength with increased bone fragility observed in both models, are likely to be mediated at least in part by enhanced androgen signaling in mature osteoblasts/osteocytes.

Thus, androgen action in the mature mineralizing osteoblast results in reduced bone biomechanics and matrix quality, with envelope-specific effects on bone formation centered on periosteal expansion. Compared with AR3.6-transgene overexpression throughout the osteoblast lineage (including bone marrow stromal cells, throughout osteoblast differentiation including osteocytes), AR2.3-transgenic males do not show reduction in femur length and demonstrate much less inhibition of whole bone strength properties including changes in stiffness, maximum load and work. However, AR2.3-transgenic mice show similar changes indicative of low turnover, and similar increases in brittleness (decreased post-yield deflection), suggesting an analogous change in matrix quality and/or mineralization in both AR3.6-transgenic and AR2.3-transgenic mice. In addition, as we have published, androgen enhances osteoblast apoptosis (14).

The most striking contrast between the two AR-transgenic models we have developed is observed at periosteal surfaces in AR3.6-transgenic males, which show increased cortical bone formation in the periosteum and dramatic intramembranous calvarial thickening. This finding was expected, given col3.6 transgene targeting to the periosteum and, conversely, the lack of expression at the same compartment with col2.3 transgene expression. The specificity of the periosteal anabolic effect in AR3.6-transgenic males is consistent with previous reports documenting the importance of androgen signaling in periosteal expansion (15). Periosteal bone formation defines the cross sectional area of bone or bone width, whereas endosteal formation or resorption determines cortical thickness. During development, girls and boys build mechanically functional structures (i.e., the size, shape and quality of the bone appears to be well matched for the size of the individual). However, they get there by different means (16). Before puberty, boys and girls grow in much the same way but during/after puberty, the increase in estrogen in girls leads to reduced periosteal expansion and then a reversal on the endosteum from expansion to infilling. In boys, testosterone levels increase and in contrast to girls, lead to further expansion of the periosteum but also continued expansion of the endosteal cavity. Consequently, the outer diameter of girls' bones tends to be smaller than that of boys' bones and greater cross sectional area is observed in males (17), yet cortical thickness is similar between males and females (18, but see 19) because of adaptive infilling in females. Thus, we propose that androgen inhibition of medullary bone formation at the endosteal surface

in males may subserve an important physiological adaptive function, being key for appropriate spatial distribution and maintenance of the total amount/weight of bone in the cortical envelope. A reasonable hypothesis is that androgens strongly promote the addition of cortical width through periosteal growth, but balance that growth with inhibition in the marrow cavity so that the skeleton does not become too heavy (see 20). Based on our characterization of AR-transgenic mouse models and other published reports, ***we propose a model for the consequences of androgen signaling where the effects of AR activation are distinct in different skeletal compartments (Fig. 7).*** Thus, in trabecular bone, androgens reduce bone turnover but increase trabecular volume through an increase in trabecular number. In cortical bone, androgens inhibit osteogenesis at endosteal surfaces but increase bone formation at periosteal sites (12), to maintain cortical thickness yet displace bone further away from the neutral axis in males. Androgens also positively influence bone at intramembranous sites (12, 21). In addition, androgen administration increases muscle mass, partially mediated by effects on mesenchymal stem cell lineage commitment (22), likely to indirectly influence bone density through biomechanical linkage.

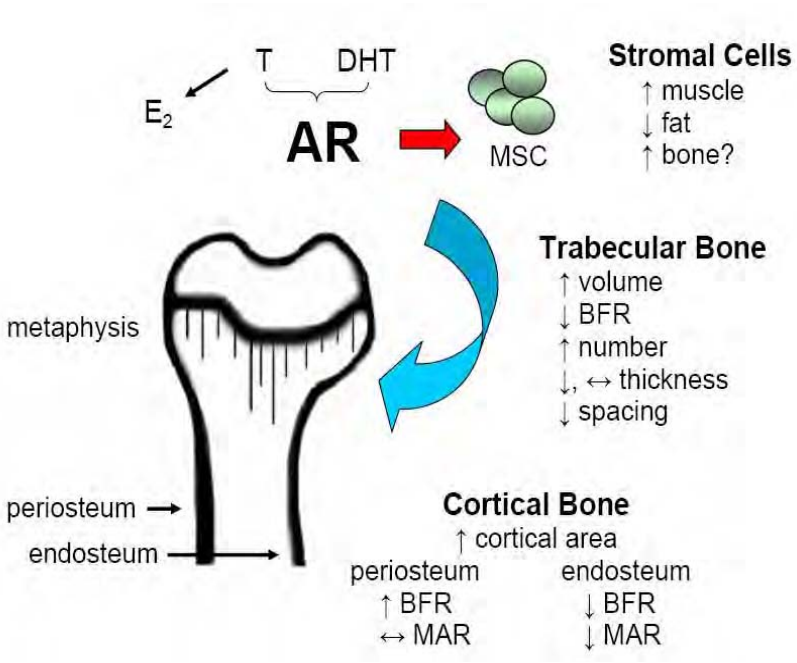


Figure 7. Model for androgen action in the skeleton mediated by AR transactivation. Androgen activation of AR influences a variety of target organs and skeletal sites, including marrow stromal cells, and trabecular, cortical and intramembranous bone compartments. Arrows indicate the changes associated with androgen action. In trabecular bone, androgen action preserves or increases trabecular number, has little effect on trabecular thickness, and thus reduces trabecular spacing. In cortical bone, AR activation results in reduced bone formation at the endosteal surface but stimulation of bone formation at the periosteal surface; correspondingly decreased periosteal but increased endosteal resorption results in no change in cortical area. In the transgenic model, AR activation in mature bone cells *in vivo* results in a low turnover phenotype, with inhibition of bone formation and inhibition of gene expression in both osteoblasts and osteoclasts. In the absence of compensatory changes at the periosteal surface, these changes are detrimental to overall matrix quality, biomechanics and whole bone strength.

To summarize, complex skeletal analysis using morphological characterization by µCT, dynamic and static histomorphometric analysis, DXA, biomechanical testing and gene expression studies all indicate that androgen signaling in mature osteoblasts inhibits osteogenesis at endosteal surfaces and produces a low turnover state; these changes are detrimental to overall matrix quality, biomechanical competence, bone fragility and whole bone strength. It is possible that the observed inhibition of osteogenesis and lack of anabolic response, as a consequence of enhanced androgen signaling in mature bone cells, underscores an important physiological function for androgen in the skeleton: to maintain an appropriate spatial distribution of bone in the cortical envelope. The strong inhibition of bone formation at the endosteal surface and increase in bone fragility may also underlie the limited therapeutic benefits observed with androgen therapy, as noted above. Because of the

detrimental consequences of direct androgen signaling in bone, these results raise concerns regarding anabolic steroid abuse or high dose androgen therapy during growth and in healthy eugonadal adults.

Male AR-transgenic mice also demonstrate a phenotype consistent with ***reduced osteoclast resorptive activity***. TRAP and RANKL expression is reduced, with an increase in OPG, an important negative regulator of osteoclast differentiation, survival and activation (13). In addition, the increase in trabecular bone volume with a decrease in trabecular separation observed is a hallmark of antiresorptive activity. Potential modulation of osteoclast action by DHT is incompletely characterized, although there are reports of AR expression in the osteoclast (23). The effect of androgen is undoubtedly complex, given data that androgens may inhibit levels of OPG (24, 25), although previously we (12) and others (26) have shown that androgen can stimulate OPG levels. Although androgen may be a less significant determinant of bone resorption *in vivo* than estrogen (27), this remains controversial (28). The bone phenotype that develops in a global AR null male mouse model, a high-turnover osteopenia with reduced trabecular bone volume and a stimulatory effect on osteoclast activity (29-31), also supports the importance of androgen signaling through the AR to influence resorption, and is generally opposite to the phenotype we observe with targeted AR overexpression. Interestingly, the global AR null model also develops late onset obesity (32). Finally, recent publications document that androgen reduces bone resorption of isolated osteoclasts (33), inhibits osteoclast formation stimulated by PTH (34), and may play a direct role regulating aspects of osteoclast activity in conditional AR null mice (35). Our results suggest that at least some component of inhibition of osteoclastic resorptive activity as a consequence of androgen administration is mediated indirectly through effects on mature osteoblasts and osteocytes. These findings reinforce the ***significance of Specific aim 2 to determine the importance of androgen in mediating osteoclast formation and activation*** (see below, Fig.16).

One final observation of note is that some of the ***negative consequences of AR overexpression in mature osteoblasts we have observed in vivo are consistent with our in vitro analyses***. For example, there are reports, some in clonal osteoblastic cell lines, of effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation mediated by AR signaling (36-38). These findings are variable however, with other reports of no effect or even inhibition of osteoblast markers (39-41), consistent with our gene expression analysis in AR-transgenic mice. In addition, the effect of androgens on osteoblast proliferation is controversial. We have previously demonstrated that either stimulation or inhibition of osteoblast viability by androgen can be observed, and these effects are dependent on the length of treatment. Transient administration of nonaromatizable DHT can enhance transcription factor activation and osteoblast proliferation, while chronic treatment inhibits both mitogenic signaling and MAP kinase activity (42). Chronic DHT treatment *in vitro* also results in enhanced osteoblast apoptosis (14). In addition, chronic DHT treatment alters distinct signaling pathways in osteocytes, as shown below in Fig. 21 and Table 1. Thus, these *in vitro* reports are consistent with the detrimental changes in matrix quality and osteoblast vigor we observe in the AR-transgenic model *in vivo*.

1b) Analysis of bone parameters with DHT replacement in gonadecomized AR-transgenic mice.

With the loss of gonadal steroids (during menopause, andropause or surgical castration), bone turnover is increased dramatically (including increased bone resorption) such that bone strength is reduced. This high turnover state does not persist however, and after ~1 year in humans (or ~2 months in mice), turnover rates nearly return to baseline. We have begun studies to examine two aspects of the phenotype we have observed in developing AR3.6-transgenic mice: inhibition of bone resorption on trabecular surfaces and mild stimulation at

periosteal surfaces and in calvaria, reflecting the known physiological effects/roles of androgens. The hypothesis tested is that DHT transactivation of AR will protect against hypogonadal bone loss following gonadecomy [either ovariectomy (OVX) or orchidectomy (ORX)] in both female and male mice.

Two experimental approaches have been taken to separately test for anti-resorptive and anabolic actions of androgen replacement in the adult in both AR2.3-transgenic and AR3.6-transgenic mice. In the first, protracted hormone ablation was followed by steroid replacement. Both male and female wild-type control (B6D2F2) and AR-transgenic mice were sham operated or gonadectomized at 3 months of age, and the effect of nonaromatizable dihydrotestosterone (DHT) or placebo was determined after an 8-week delay, allowing for gonadectomy-induced changes to develop. In this setting, hormone administration can be considered as a therapeutic measure. Following 6 weeks of treatment, the effects of androgen on bone and whole body composition was assessed by DXA. The second approach is characterized as preventative. In the second paradigm, hormone ablation as a consequence of gonadecomy at 5 months of age was followed immediately by steroid replacement, again for 6 weeks. Thus, both groups of animals were analyzed at 6.5 months of age.

We have focused on characterization of the anabolic (increased bone formation) effects in male and female mice castrated at ~3 months (initially in wild-type littermate controls) with steroid pellet replacement delayed for 2 months to allow turnover to stabilize. DHT was delivered for ~6 weeks, and mice were then evaluated for changes in bone mineral by dual energy x-ray absorptiometry (DXA) using a mouse PIXImus2 densitometer. We presented preliminary results last year, and have now nearly finished the DXA arm of the study (with a few animals remaining to be analyzed), with histomorphometric analysis now being completed. We have also nearly finished with surgeries and steroid replacement in the 6.5 month old high-turnover, prevention studies. DXA has been performed on ~ half of these mice.

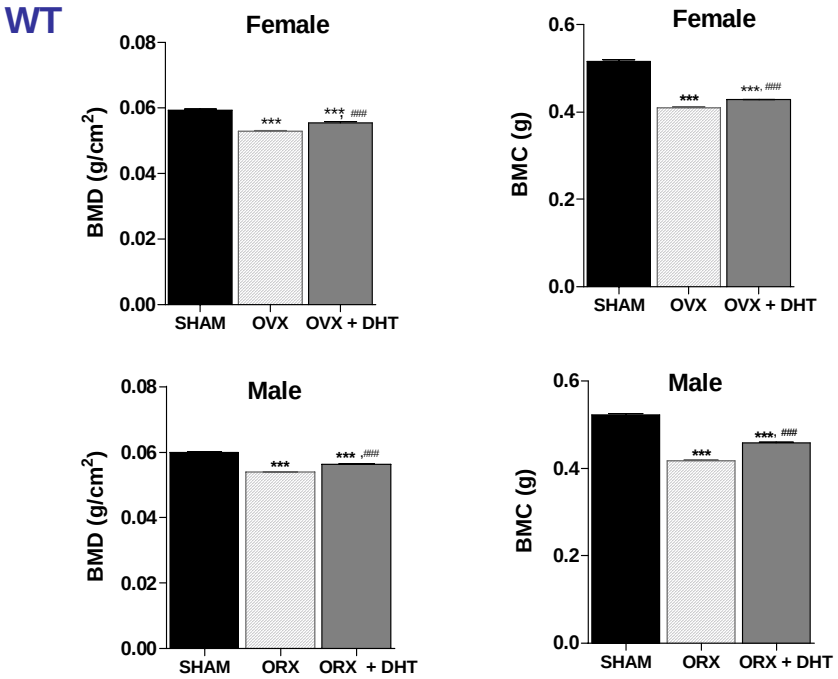


Figure 8. DXA analysis of gonadectomized, wild-type (wt) mice treated with placebo or DHT. Female (n = 18-34) BMD and BMC (*top panels*) and male (n = 26-30) BMD, BMC (*bottom panels*). One-way ANOVA s reveal a significant difference of $p < 0.001$. Tukey's multiple comparison test's show significant differences between SHAM and OVX/ORX mice in both genders (***, $p < 0.001$) and between DHT administration vs gonadectomized mice (###, $p < 0.001$).

In ***wild-type animals***, ovariectomy (OVX) in females (n = 14-17) resulted in significant decreases in BMD (13.8%, $p < 0.001$) and BMC (22%, $p < 0.001$) compared to sham, consistent with other reports. Systemic DHT administration improved BMD and BMC in these mice compared to placebo-treated animals (n = 14-26). In males, orchidectomy (ORX) also resulted in reduced BMD and BMC (10.5%, $p < 0.001$ and 20.3%, $p < 0.001$, respectively) and 6 weeks of DHT treatment was sufficient to improve or fully restore these deficits in bone mineral. These results suggest that androgen replacement in hypogonadal populations (either male or female) can be an effective therapy for bone mineral loss.

AR3.6

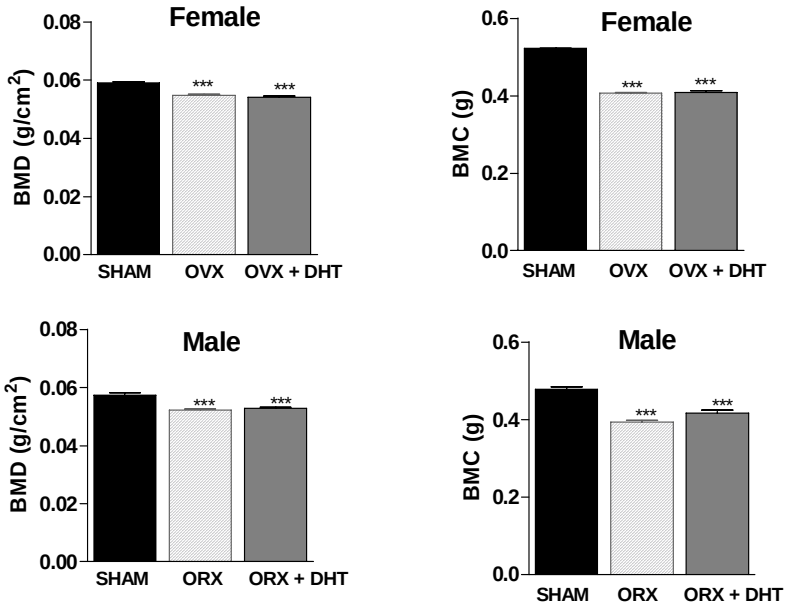


Figure 9. DXA analysis of gonadectomized AR3.6-tg mice treated with placebo or DHT. Female (n = 8-15) BMD and BMC (*top panels*) and male (n = 5-8) BMD, BMC (*bottom panels*). One-way ANOVAs reveal a significant difference of $P < 0.001$. Tukey's multiple comparison test's show significant differences between SHAM and OVX/ORX mice in both genders (**, $p < 0.01$; ***, $p < 0.001$), but no significant difference between DHT administration vs gonadectomized mice.

Although gonadectomy in skeletally-targeted ***AR-transgenic mice*** also resulted in a loss of bone mineral, the response to DHT treatment was blunted compared to wild-type. In female AR2.3-transgenic mice a response similar to wild type was observed with BMD decreasing 22.2% ($p < 0.001$) and BMC reduced 24.1% ($p < 0.001$) with OVX, yet DHT treatment did not improve either bone measure when compared to placebo-treated OVX mice (n = 3-14). In AR3.6-transgenic female mice (n = 8-14), BMD loss following OVX was less dramatic (7.6 %) but still highly significant ($p < 0.001$), while the decrease in BMC was nearly 24% ($p < 0.001$). Similarly, in male AR3.6-transgenic mice (n = 5-7), ORX decreased BMD by 5.1% ($p < 0.05$) while a 15.1% ($p < 0.001$) loss in BMC was observed. Again, as in AR2.3-transgenic females, DHT administration to AR3.6-transgenic mice of either sex did not reverse the loss in bone mineral as observed in wild type, littermate controls.

We have also determined the effect of 6 weeks of DHT treatment in ***intact male and female wild-type mice***. Surprisingly, eugonadal females lost 4.8 % BMD ($p < 0.05$) and 8.9% BMC ($p < 0.05$) and although male mice did not lose BMD, a 4.1% ($p < 0.001$) decrease in BMC resulted. Feedback regulation of the hypothalamic-pituitary-gonadal axis may partially underlie these results, and we are exploring potential mediators.

AR2.3

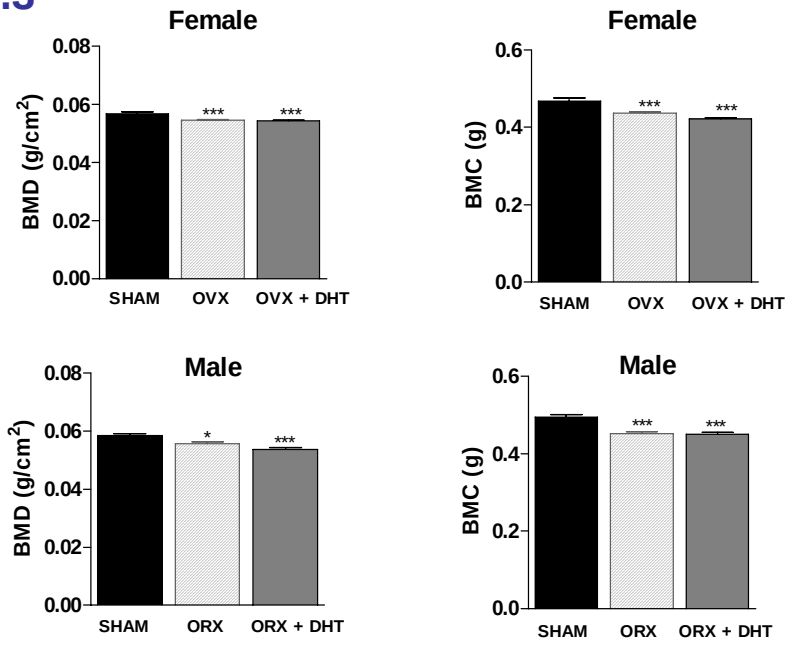


Figure 10. DXA analysis of gonadectomized AR2.3-tg mice treated with placebo or DHT. Female (n = 8-16) BMD and BMC (top panels) and male (n = 6-12) BMD, BMC (*bottom panels*). One-way ANOVA s reveal a significant difference of $P < 0.001$. Tukey's multiple comparison test's show significant differences between SHAM and OVX/ORX mice in both genders (*, $p < 0.05$; ***, $p < 0.001$), but no significant difference between DHT administration vs gonadectomized mice.

Intact WT

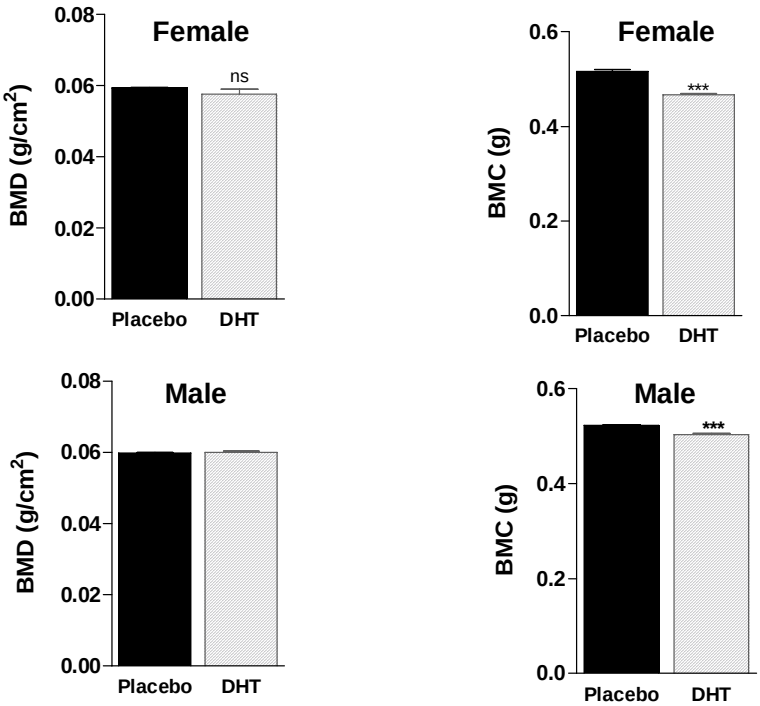


Figure 11. Bone mineral measures from intact wild-type (wt) mice treated with placebo or DHT. Female (n = 18-34) BMD and BMC (*top panels*) and male (n =26-30) BMD, BMC (*bottom panels*). Statistical analyses by unpaired t-test revealed that no significant differences in BMD were seen in either sex in response to DHT, however, BMC was reduced in both sexes (***, $p < 0.001$).

Summary of findings for bone mineral analyses:

- Nonaromatizable androgen (DHT) effectively treats hypogonadal bone loss in wild-type male and female mice following OVX/ORX.
- AR2.3-transgenic male and female mice lose bone mineral following gonadectomy, yet do not improve with DHT treatment.
- AR3.6-transgenic mice also lose bone mineral following gonadectomy, and female mice do not improve with DHT treatment. Males may show slight improvements in BMC.
- DHT administration to intact wild-type mice resulted in reduced bone mineral content.

Combined, these data indicate that systemic DHT administration positively influences bone mineral changes only after a prolonged hypogonadal state in both male and female wild-type controls. Notably, enhanced sensitivity to androgen through AR overexpression in either the mature bone compartment (AR2.3) or in the stromal compartment and proliferating osteoblasts (AR3.6) abrogates the positive effects of DHT on bone mineral accretion, suggesting that bone itself is not a positive *direct* therapeutic target.

1c) Analysis of body compositional changes gonadectomized mice.

Androgens have well characterized anabolic effects on muscle mass and strength. In the course of characterization of bone mineral changes in AR3.6-transgenic mice, we noted that male AR3.6-transgenic mice also demonstrate a body composition phenotype, with decreased fat mass but increased lean mass. We have proposed the hypothesis that this change in body composition is a consequence of AR expression in stromal precursors; pluripotential cells with the ability to form a variety of tissues including muscle, fat, bone, cartilage as outlined in the schema for AR2.3 and AR3.6 expression patterns shown above. We have shown AR3.6-transgene expression in bone marrow stromal cells. With AR transactivation in male mice during development, we propose that bone marrow stromal cells respond by alteration of lineage commitment, away from the adipocyte and instead toward the myoblast to form more muscle in the AR3.6-transgenic mice. DXA analysis has been employed to characterize body compositional changes that occur in the adult setting after gonadecomy and DHT replacement.

The two experimental paradigms described for bone mineral analysis were also employed for body composition analysis. Anabolic (increased lean mass) effects were examined in male and female mice castrated at 3 months (initially in wild-type littermate controls) with steroid pellet replacement delayed for 2 months to allow turnover to stabilize. DHT was delivered for 6 weeks, and mice were then evaluated for changes in body composition by DXA in this therapeutic arm. We also performed analysis in a preventative paradigm, with replacement given immediately after gonadecomy.

Gonadectomy has been shown to reduce lean mass and increase fat as a percent of body weight in humans and rodent models. We observed a similar trend in ***wild-type*** females (n = 14-26) after OVX with increased fat (12.8%) and reduced lean mass (5.3%), although the data did not reach statistical significance. In addition, modest weight gain was seen in wild type females as a consequence of OVX (3.4%, ns). However, unlike males, female mice were ***completely resistant*** to the positive effects of DHT to restore body composition (either fat or lean mass) over the six-week treatment period. Our results also show that following ORX, wild-type males (n = 14-23) demonstrate a significant increase in fat mass as a percent of body weight (16.5 %, $p < 0.05$). Conversely, the percent lean mass was reduced (7.6%, $p < 0.05$) and a small but non-significant decrease in body weight (3.5%) was observed compared to sham controls. In this setting, DHT treatment was beneficial, improving or fully restoring body composition changes induced by ORX compared to placebo. These results suggest that

WT

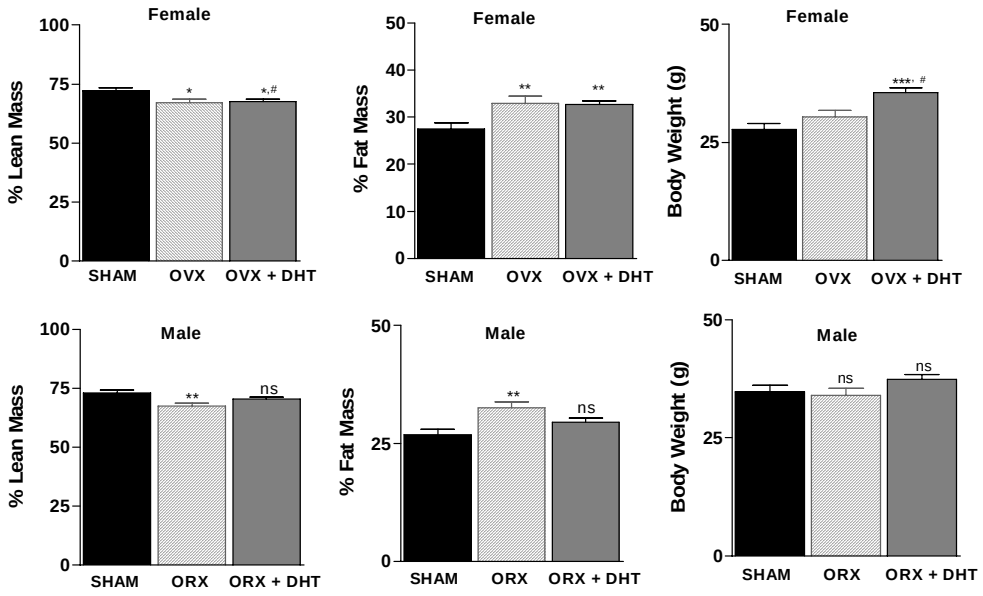


Figure 12. Body composition analysis of gonadectomized wild-type (wt) mice treated with placebo or DHT. Female (n = 18 - 34) percent lean mass, percent fat mass, and total body weight (*top*) and male (n = 26 - 30) percent lean mass, percent fat mass, and total body weight (*bottom*). Female mice lost lean mass (*, $p < 0.05$) and gained fat mass (**, $p < 0.01$) following OVX and DHT treatment caused weight gain above both sham ($p < 0.05$, *) and OVX (***, $p < 0.001$) groups. Males lost lean mass (**, $p < 0.01$) and gained fat mass (**, $p < 0.01$), but showed no significant changes in body weight.

AR3.6-tg

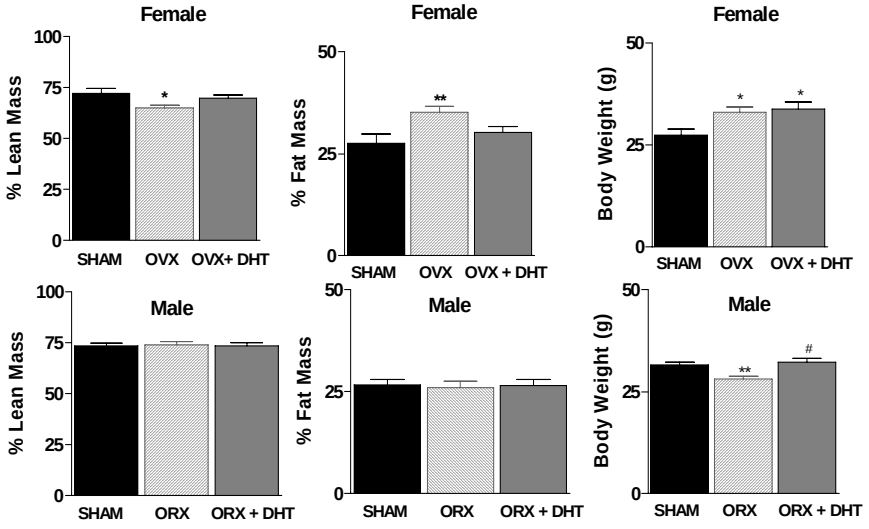


Figure 13. Body composition analysis of gonadectomized AR3.6-tg mice treated with placebo or DHT. Female (n = 8-15) percent lean mass, percent fat mass, and total body weight (*top*) and male (n = 5-8) percent lean mass, percent fat mass, and total body weight (*bottom*). Females lost significant lean mass (*, $p < 0.05$), gained fat mass (**, $p < 0.01$), and body weight (**, $p > 0.01$) following OVX. In contrast, male AR3.6-tg mice did not exhibit alterations in body composition following ORX, although weight loss was observed (**, $p < 0.01$). In these mice, DHT treatment had no significant effect on body composition in either sex, however, male mice regain the weight lost following ORX ($^{\#\#}$, $p < 0.05$).

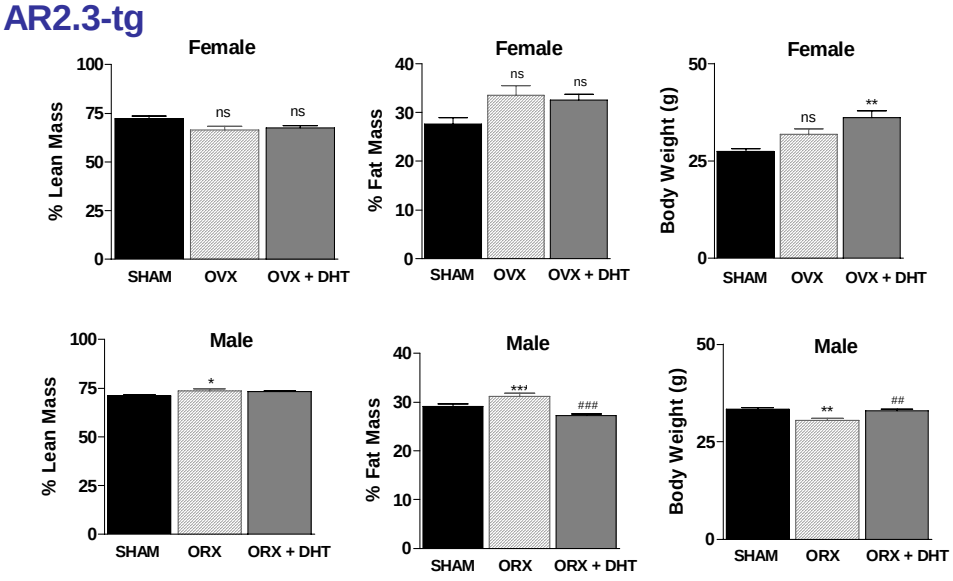


Figure 14. Body composition analysis of gonadectomized AR2.3-tg mice treated with placebo or DHT. Female (n = 8-16) percent lean mass, percent fat mass, and total body weight (*top*) and male (n = 6 -12) percent lean mass, percent fat mass, and total body weight (*bottom*). Females did not significantly lose lean mass or gain fat mass following OVX but DHT treatment did result in weight gain compared to sham (**, $p < 0.01$). Surprisingly, male mice gained lean mass (*, $p < 0.05$) and fat mass (**, $p < 0.01$) and while DHT treatment did not significantly alter lean mass, it did reduce fat mass relative to placebo ($^{###}$, $p < 0.001$). Male mice also lost body weight following ORX ($p < 0.01$, **) and DHT was able to restore this loss ($^{##}$, $p < 0.01$).

females may not be as responsive to androgen treatment as an anabolic strategy to increase lean mass.

Skeletally-targeted **AR overexpression models** show similar responses to gonadectomy. Following OVX, AR2.3-transgenic female mice (n = 3-8) develop alterations in body composition that mirrored wild types with 8.7% decreased lean mass and a 17.9% increase in fat mass. The seemingly large percent change was not statistically significant however, likely due to the small size of this cohort. Again, similar to wild-type controls, female AR2.3-transgenic mice were insensitive to six weeks of DHT administration. Male AR2.3-transgenic mice lost 3.5% lean and gained 7.9% fat mass, and although these changes were not significant, DHT treatment improved body composition to a level above sham-operated controls. In AR3.6-transgenic females (n = 8-14) lean mass decreased 10.3% ($p < 0.05$) and a 21.5% ($p < 0.05$) gain in fat mass developed by two months after OVX. In these mice, DHT administration effectively treated the changes in lean and fat mass. Intact AR3.6-transgenic males display reduced fat mass and increased lean mass as a consequence of transgene expression. In these mice, gonadectomy did not alter body composition and DHT treatment provided no further improvement to these measures.

In contrast to gonadectomized models, DHT treatment in **intact wild-type mice** had a negative impact on body composition in both males and females, analogous to losses in bone mineral. With DHT treatment, females (n = 14-26) lost 2.8% lean mass and gained 7.8% fat mass compared to placebo. A similar but more dramatic change was observed in wild-type males (n = 14-18) where a 6% ($p < 0.05$) drop in lean mass and a 13.6% ($p < 0.05$) increase in fat was observed.

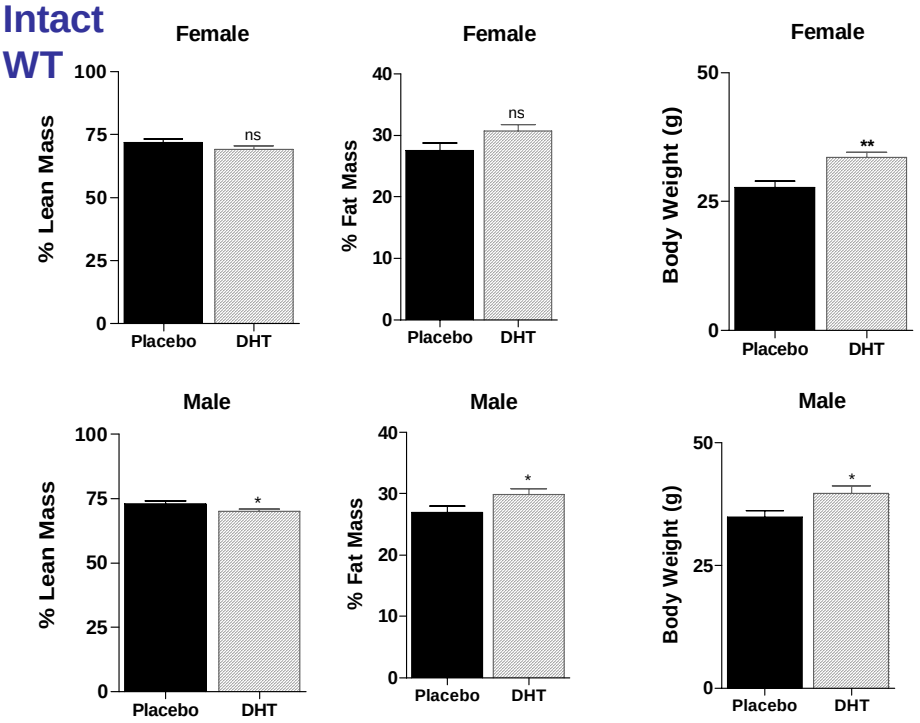


Figure 15. Body composition analysis of intact wild-type (wt) mice treated with placebo or DHT. Female (n = 18-34) percent lean mass, percent fat mass, and total body weight (*top*) and male (n = 26-30) percent lean mass, percent fat mass, and total body weight (*bottom*). Females lost significant lean mass (*, $p < 0.05$) and gained fat mass (**, $p < 0.01$) following OVX. Males similarly lost lean mass (**, $p < 0.01$) and gained fat (**, $p < 0.01$). Females showed a trend toward weight gain in response to OVX and DHT treatment resulted in significant weight gain ($p < 0.001$, ***). Males showed a trend toward weight loss following ORX and weight gain in response to DHT treatment.

Summary of findings for body compositional analyses:

- In wild-type mice, 8 weeks in a hypogonadal state results in reduced lean mass and increased fat in both sexes, and a trend toward reversal was observed following DHT treatment.
- AR3.6-transgenic mice lost lean mass and gained fat and DHT treatment showed some improvement. However, while male mice were resistant to body composition changes induced by ORX, they did lose body weight and DHT did restore this loss.
- AR2.3- transgenic mice showed similar trends in body composition changes observed following OVX/ORX, but males gained lean mass. In these mice, DHT was able to reverse most of the alterations.
- DHT administration to intact wild-type mice resulted in reduced lean mass, increased fat mass, and increased body weight in both sexes.

Combined, these data indicate that systemic DHT administration positively influences body composition changes after a prolonged hypogonadal state in males, but is ineffective in females over the same time frame. DHT treatment in the intact animal results in negative alterations in body composition and should be approached with caution when considering androgens as a therapeutic intervention.

2. Studies proposed in Specific aim 2 to determine the importance of AR in regulating osteoclast formation and activation.

2a) characterization of *ex vivo* cultures derived from AR3.6-transgenic mice.

Our initial studies have utilized *ex vivo* culture of enriched bone marrow macrophage cells from AR3.6-transgenic mice to begin characterization of the effects of androgen signaling on osteoclast formation and activation. AR3.6-transgenic mice were chosen for initial analysis because of reports of low level expression of the col3.6 promoter in osteoclastic cells *in vivo* (43). In our preliminary analysis, long bones (femur and tibiae) from 8-10 week old wild-type or

AR3.6 transgenic mice were aseptically dissected free of muscle and connective tissue and placed in a dish containing phenol red-free α-MEM with 10% charcoal-stripped (CS)-FCS and antibiotics. The epiphyses were removed with scissors and the marrow cavity was flushed with the same media using a syringe fitted with a 27 gauge needle. Marrow cells were collected by centrifugation at 250 *g* for 5 min. at 4 ° C and resuspended in erythrocyte lysis buffer [17 mM Tris-HCl, pH 7.6 and 0.8 % NH_4Cl] and stored on ice for 5 min. Ten milliliters of fresh media was added and the cells were pelleted as above. To enrich for bone marrow macrophages (BMM), cells were plated at 5 x 10^6 cells per 60 cm^2 suspension culture dish in α-MEM containing 10% FCS and antibiotics plus 100 ng/ml M-CSF and cultured for 3 days. Following another 3 days of culture in the presence of 100 ng/ml M-CSF, the cells were used in osteoclast formation assays.

To induce osteoclast formation, cells are washed with PBS, detached with trypsin/EDTA and

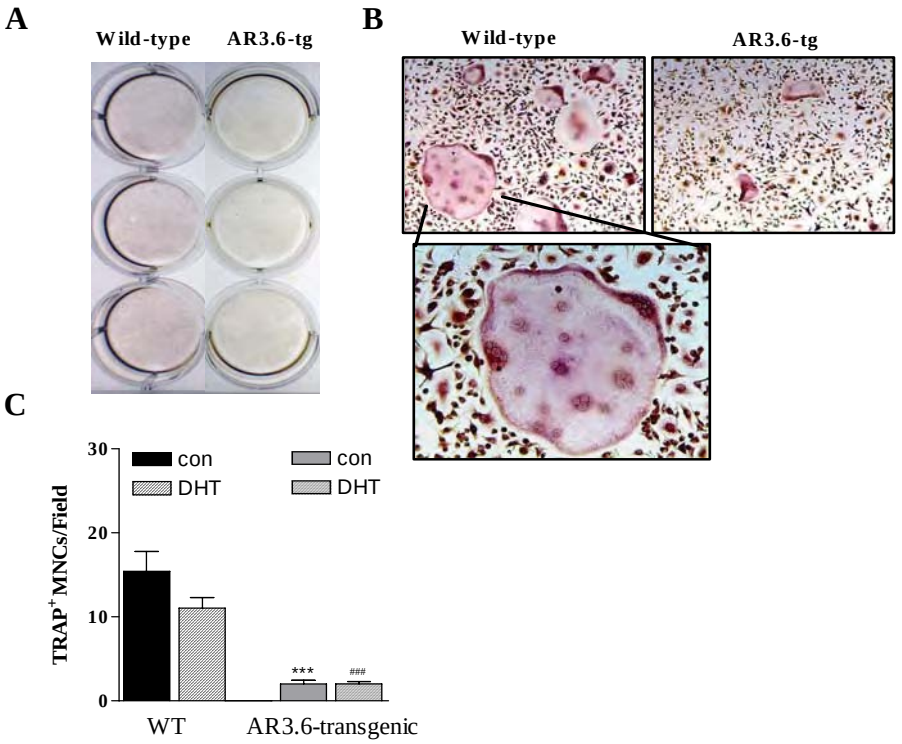


Figure 16. Analysis of osteoclastogenesis in *ex vivo* cultures from AR3.6-transgenic (AR3.6-tg) mice. Whole bone marrow was harvested and osteoclast precursors isolated as described from AR3.6-transgenic mice or wild-type controls. After induction, cells were treated with vehicle or 10^{-8}M DHT for 7-10 days. Cells were fixed and stained for TRAP activity. TRAP+ multinucleated cells (≥3 nuclei) were identified as osteoclasts. **A.** TRAP staining in wild-type versus AR-3.6 transgenic osteoclast cultures. **B**. Morphology of osteoclasts in high power image. **C**. Quantitation of TRAP+ multinucleated osteoclastic cells. Data represents the mean ± SEM of the number of $TRAP^+$ MNC in duplicate cultures. Statistical analysis was performed using *t* test. ***, $p <$ 0.001 vs. wt con; $^{###}$, p , 0.001 vs. wt DHT.

centrifuged. The cell pellets are resuspended in growth media and counted. Enriched BMM cultures are plated at a density of 50x 10^4 cells/cm^2 in 24-well plates in α-MEM plus 10 % CS-FCS containing 100 ng/ml of M-CSF and 50 ng/ml of RANKL. To determine the effects of androgen on osteoclast formation, 10^{-8} M DHT or vehicle (ethanol) is added to appropriate wells. The cells are maintained in this media for 7-10 days with media changes every 2-3 days. The cells are then fixed and stained for tartrate-resistant acid phosphatase (TRAP) following the manufacturer's instructions. Osteoclasts are counted as TRAP positive cells containing 3 or more nuclei from duplicate wells from at least 5 random fields/well.

These results suggest that in the AR3.6-transgenic model, cell autonomous osteoclast formation is inhibited. A recent study by Boban *et al* (43) has shown a very low level of col3.6 promoter activity in osteoclasts, consistent with this result. Now that we have improved our culture methods, our next steps will be to analyze autonomous osteoclast formation in AR2.3-

transgenic mice, and then to perform a mix and match co-culture analysis with both AR- transgenic osteoblastic cells (derived from marrow or calvaria) with bone marrow stromal osteoclast precursors.

3. Studies proposed in Specific aim 3 to characterize the role of androgen in regulating proliferation and differentiation in the osteoblast lineage.

3a) Characterization of proliferation and differentiation in AR2.3-transgenic calvarial osteoblast (mOB) cultures.

The direct mechanisms by which androgens influence bone cells are not fully understood. To assess the direct effects of androgen signaling during osteoblast differentiation, we are studying the effects of DHT on cell proliferation and differentiation in primary calvarial cultures (mOB) derived from both AR3.6-transgenic and AR2.3-transgenic fetal mice. Osteoblast function is assessed by determining cell DNA, alkaline phosphatase activity, and mineral deposition. In these initial studies, we have focused on AR2.3-transgenic cultures with overexpression in mature osteoblasts/osteocytes because endogenous AR levels are highest in osteocytes and because the comparison in phenotype between AR2.3 and AR3.6-transgenic mice suggests that mature osteoblasts osteocytes are important mediators of androgen action in the skeleton, as noted above.

Osteoblastic cells were isolated after collagenase digestion from fetal calvaria from both wild-type and AR2.3-transgenic fetal mice. Briefly, calvariae were isolated from 3-6-day-old mice after genotyping. The parietal and occipital bones were dissected free of the suture lines and subjected to four sequential 15-minute digestions in a mixture containing 0.05% trypsin and 0.1% collagenase-P at 37ºC. Cell fractions 2-4 were pooled and plated at 8000 cells/cm^2 in MEM supplemented with 10% FBS. Beginning at day 7, cultures were switched to differentiation medium in phenol-red free BGJb (Fitton-Jackson modification) supplemented with 10% charcoal-stripped FBS containing 50 µg/ml ascorbic acid. From day 14 on, 5 mM ß-glycerophosphate was added to the differentiation media.

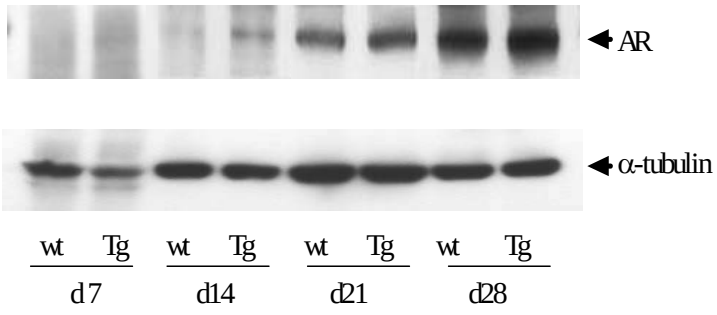


Figure 17. Western analysis of AR abundance in *ex vivo* primary osteoblast cultures from AR2.3-transgenic (AR2.3-tg) mice. Cell extracts were separated on 10% SDS-polyacrylamide gels and AR protein levels were analyzed by Western blotting using polyclonal anti-AR antibody. The upper panel shows the AR levels detected in cell lysates of both wt and AR2.3-Tg cells at indicated time points. The typical AR is indicated by the *arrow* at 110 kDa. Differentiation of primary calvarial cells shows increasing intensity of AR levels over time. The lower panel shows lysates reprobed with α-tubulin antibody in order to assess consistency of protein loading.

As shown in Fig. 17, we first determined the level of AR abundance in both wild-type and AR2.3-transgenic cultures by Western analysis, with polyclonal rabbit AR antibodies (ARN-20) purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and used at 1:300. The α-tubulin antibody (T9026) was a mouse monoclonal antibody purchased from Sigma and was used at 1:1000. The membrane was incubated with either horseradish peroxidase-linked goat anti-rabbit IgG antibody (Bio-Rad Laboratories, Hercules, CA,USA) or goat anti-mouse IgG antibody (Bio-Rad) at 1:2000 for 1 h. Bound antibodies were visualized by enhanced

chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) on Kodak X-AR5 autoradiographic film. As expected, AR levels increase during differentiation in both cultures. The highest level of AR overexpression noted after day 21 in AR2.3-transgenic cultures. We then assessed growth characteristics of wild-type and AR2.3-transgenic cultures without hormone treatment. As shown in Fig.18, osteoblastic cells from both transgenic and wild-type mice display similar growth kinetics. We also determined mineralization capacity (see Fig.19), and observed no significant difference between AR2.3-transgenic versus wild-type cultures without hormone addition.

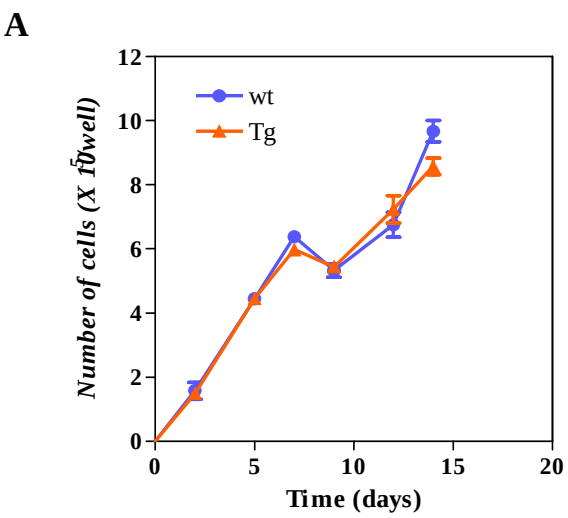


Figure 18. Analysis of osteoblast proliferation in *ex vivo* cultures from AR2.3-transgenic (AR2.3-tg) mice. Osteoblast-enriched primary cultures were derived from fetal calvaria by collagenase digestion. Cells were counted using a Coulter Counter. There was no significant difference in growth characteristics between wild-type and AR2.3-transgenic osteoblastic cells.

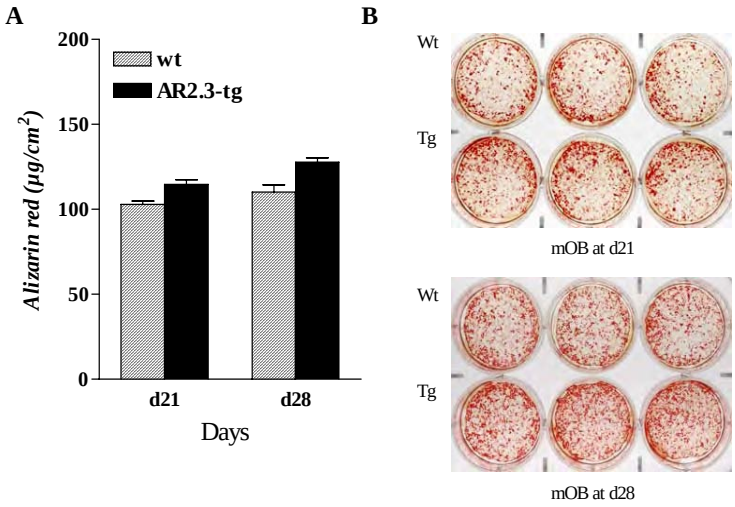


Figure 19. Analysis of osteoblast mineralization in *ex vivo* cultures from AR2.3-transgenic (AR2.3-tg) mice. Osteoblast-enriched primary cultures were derived from fetal calvaria by collagenase digestion. Cells were grown for 21 or 28 days, and mineralization assessed by alizarin red assay (**A**) or staining (**B**). There was no significant difference in mineral apposition between wild-type and AR2.3-transgenic osteoblastic cells.

Finally, we characterized alkaline phosphatase, an osteoblast marker activity (Fig.20). Cultures were treated continuously with vehicle or 10^{-8}M DHT (a nonaromatizable androgen) in charcoal-stripped serum. Analysis for alkaline phosphatase activity was performed using *p*-nitrophenylphosphate as substrate. Absorbance was read at 405 nm using a microplate reader. Alkaline phosphatase activity was expressed as nmol *p*-nitrophenol released per min per µg protein. All analyses were done in six replicates at each time. Each experiment was repeated three to four times. Cultures treated with DHT showed significant inhibition in alkaline phosphatase activity, with the most robust inhibition observed in AR2.3-transgenic cultures. These results are consistent with a reduction in osteoblast differentiation and may reflect the reduced osteoblast vigor in AR2.3-transgenic mice as described above.

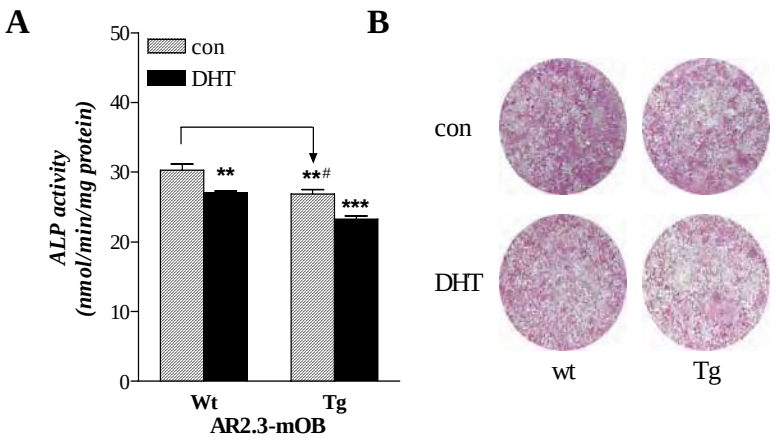


Fig. 20. Characterization of changes in alkaline phosphatase (ALP) in differentiating primary calvarial osteoblasts. A. ALP activity. Changes in alkaline phosphatase were measured after DHT treatment for 14 days in both wt and AR2.3-Tg calvarial osteoblasts. Data are expressed as mean ± SEM of six repeats. **, $p < 0.01$; ***, $p < 0.001$ compared to exposure to vehicle; **#, $p < 0.01$ compared to wt control. **B.** AP staining in osteoblastic cultures.

3b) Global profiling analyses of the effects of androgen in osteocytes using microarray analysis.

Given that the osteocyte is the most abundant cell type in bone, and that osteocytes have the highest level of endogenous AR expression which suggests that they are an androgen target, we performed gene profiling analysis for transcripts regulated by chronic DHT treatment. We examined the effect of chronic DHT treatment in primary cultures of calvarial osteoblasts. Expression analysis was performed with samples isolated after 26 days of 10^{-8}M DHT or vehicle treatment, after hybridization of complex probes to Research Genetics cDNA arrays. We used an arbitrary cut-off of 1.5-fold regulation based on our previous experience with cDNA arrays in which we have confirmed by qRT-PCR analysis ~80% of the regulated genes identified (44).

Table 1. Top androgen regulated transcripts.

Symbol	Name	Accession	Regulation
	DHT Decreased:		
SPP1	osteopontin	AA775616	-1.49
COX6A1	cytochrome c oxidase subunit VIa polypeptide 1	NM_004373	-1.28
GAPDH	glyceraldehyde-3-phosphate dehydrogenase 1	NM_002046	-1.25
MAN2A2	mannosidase, alpha, class 2A, member 2	NM_006122	-1.14
AKR1C1	aldo-keto reductase family 1, member C1	NM_001353	-1.12
CCNA2	cyclin A2	NM_001237	-0.97
NME1	non-metastatic cells 1, protein	NM_198175	-0.95
RPL10A	ribosomal protein L10a	NM_007104	-0.93
LDHA	lactate dehydrogenase A	NM_005566	-0.93
IFI35	interferon-induced protein 35	NM_005533	-0.91
	DHT Increased:		
KLK6	kallikrein 6	NM_002774	1.31
HLA-DMB	major histocompatibility complex, class II, DM beta	NM_002118	1.35

RNF5	ring finger protein 5	BX648378	1.35
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	NM_004996	1.35
KCNB1	voltage-gated potassium channel, Shab-related subfamily, member 1associated 1	NM_004975	1.37
CHN2	Chimerin 2	NM_004067	1.38
TSG101	tumor susceptibility gene 101	NM_006292	1.41
ESR1	estrogen receptor 1, ERalpha	NM_000125	1.42
ELA3A	elastase 3A	NM_005747	1.43
NFIL3	nuclear factor, interleukin 3 regulated	NM_005384	1.44

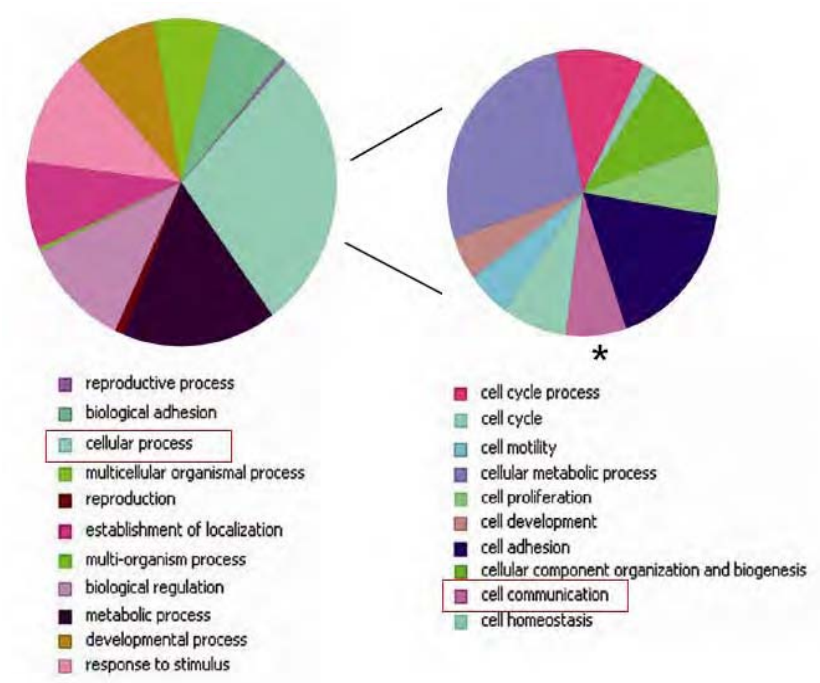
The ten most highly androgen up or downregulated named sequences were identified from the list of 375 significantly regulated transcripts. Transcripts are listed in ascending order. Regulation represents log 2 fold-change of DHT vs. control.

A total of 509 DHT up and downregulated genes were identified in these osteocytic primary cultures. The 20 most up or downregulated named sequences are shown in Table 1. Several genes involved in PI3 kinase/Akt signaling, metabolic enzyme activity and modulation of stem cell differentiation are listed. Also of note, ABCC1 is implicated in mineralization, AKR1C1 inhibits metabolism of DHT, and mannosidaseα 2A 2 is inhibited; deficiency is associated with "thickened calvaria, ovoid configuration, flattening and hook-shaped deformity of the vertebral bodies, hypoplasia of the inferior portions of the ilia, and mild expansion of the short tubular bones of the hands" (45). These specific gene expression patterns in osteocytes suggest chronic androgen may mediate inhibition of osteogenesis and a reduction in some components of the extracellular matrix, consistent with the reduction in osteoblast vigor shown in AR2.3-transgenic male mice.

To characterize meaningful signaling pathways that were impacted by DHT in mature osteoblasts/osteocytes in addition to identification of specific genes, computational characterization with GO database analysis was used to group significantly regulated named genes (375 of the 509 genes) into similar biological or molecular functional categories. For this analysis, we employed Pathway Architect software to identify GO groups statistically over-represented in the class of regulated sequences, based on frequency of expression in the category. For visualization, specific signaling categories are shown in Fig. 21A. Examination of the results revealed several notable findings; here we have focused on the category of "cell surface signaling", identified as targeted by androgen (Fig. 21B). For example, upregulatinon of PDGF receptor A would be predicted to inhibit osteoblast differentiation (46). Again, these results are consistent with a lack of anabolic action in bone.

Summary of important results: The specific role of the AR in maintenance of skeletal homoeostasis remains controversial. To determine the specific physiologic relevance of androgen action in the mature osteoblast/osteocyte population in bone, mice with targeted AR overexpression in mature osteoblasts were developed to compare and contrast with transgenic lines with AR overexpression in stromal cells and throughout the osteoblast lineage. Characterization of the consequences of bone-targeted overexpression revealed a skeletal phenotype in male transgenic mice versus littermate controls, with little difference between the females. Collectively, the phenotype observed in male transgenic mice is likely dependent on the higher levels of androgen (~10-fold) circulating in males *vs*. females. In these studies we have found that AR overexpression in the mature osteoblast population *in vivo* results in a low turnover state with increased trabecular bone volume, but a significant reduction in cortical bone area due to inhibition of bone formation at the endosteal surface and a lack of marrow infilling.

A



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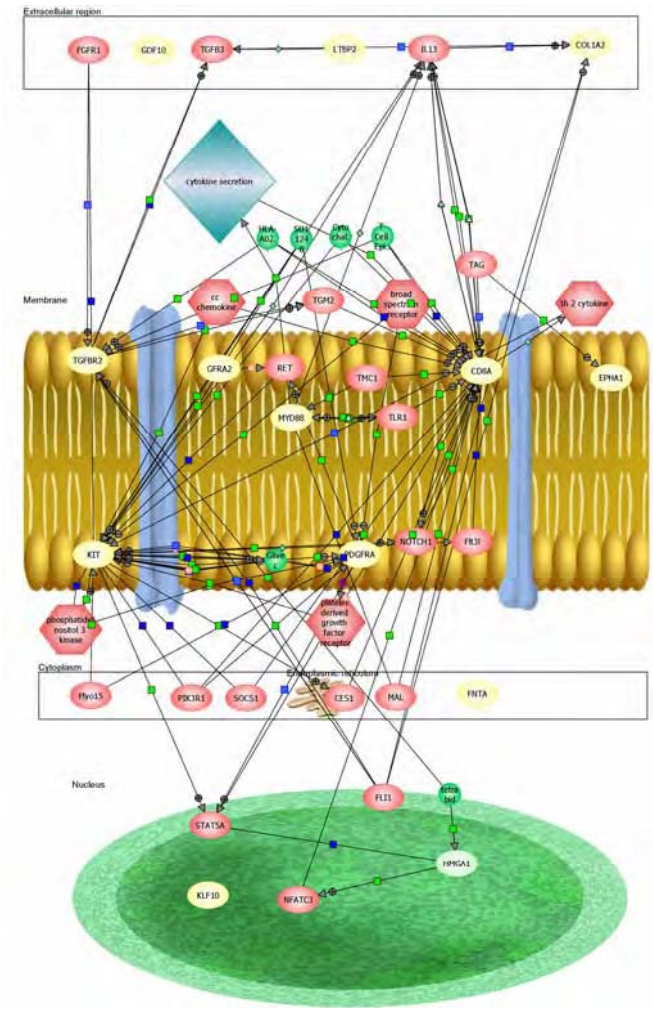

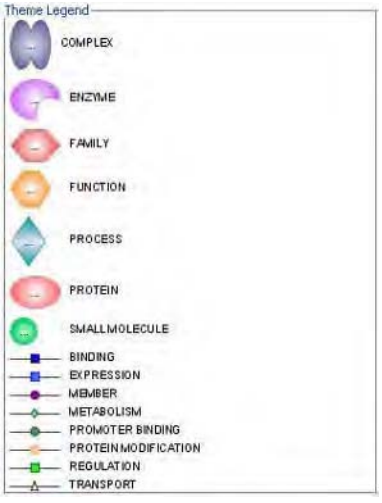

Figure 21. Bioinformatic analyses of androgen-regulated gene expression in osteocytes cultures. A. Summary of androgen-regulated GO categories. The 375 of 509 regulated genes with known functions were classified according to their gene ontology annotation terms for each group. Several GO categories were identified as significantly over-represented in the population based on frequency in the category using Pathway Architect analysis. Additional GO categories are displayed after expansion from the cellular process category. We have focused here on cell communication. **B**. **Characterization of interacting networks in the biological pathway cell surface signaling, within the category of cell communication**. Theme images are described in the legend. Upregulated transcripts are yellow; downregulated are light green. Known protein interactions are displayed in red. Downregulated architectural transcription factor HMGA1 is involved in regulation of genes and may normally promote proliferation; upregulation of PDGF receptor would be predicted to inhibit osteoblast differentiation as discussed in text.

Every measure of biomechanical responsiveness is significantly inhibited. The most remarkable aspect of the phenotype is a dramatic reduction in osteoblast vigor. *Ex vivo* analysis of osteoblast differentiation in primary culture suggests that androgen directly inhibits differentiation, while osteoclastogenesis is also directly inhibited, consistent with the low turnover phenotype. Thus, primary outcomes of analyses through year three demonstrate that enhanced androgen signaling in mature osteoblasts/osteocytes inhibits cortical bone formation, and results in changes that are detrimental to matrix quality, biomechanical competence and whole bone strength. Reductions in osteoblast vigor present *in vivo* likely reflect inhibition of osteoblast differentiation observed *in vitro* in mOB cultures and in pathways identified in gene profiling analysis. Since osteocytes are the most abundant cell type in bone, and AR levels are highest in osteocytes, these cells are a likely target for androgen signaling in bone. Gene expression profiling analysis suggests that osteocytes respond to chronic DHT treatment by modulation of signaling pathways that highlight the importance of PDGF and TGFß signaling, apoptosis, and modulation of signal transduction through membrane receptors, particularly Akt and Wnt pathways.

Key Research Accomplishments

Accomplishments for 2006-2007 directly characterizing androgen action in the skeleton:

Peer reviewed publications:

- Bi LX, **Wiren KM**, Zhang X-W, Oliveira GV, Klein GL, Mainous EG, Herndon DN: Direct effect of oxandrolone treatment on human osteoblastic cells is modest. J Burns Wounds 6:53-64, 2007
- Semirale AA, **Wiren KM**: Androgen administration has therapeutic advantages in the hypogonadal, but should be approached with caution in healthy adults. Musculoskel Neuron Interact *in press*, 2007
- **Wiren KM**, Semirale AA, Zhang X-W, Woo A, Tommasini SM, Price C, Schaffler M, Jepsen KJ: Targeting of androgen receptor in bone reveals a lack of anabolic action and inhibition of osteogenesis. A model for compartment-specific androgen action in the skeleton. FASEB J *in review*, 2007

Abstracts:

- Zhang X-W, Semirale A, **Wiren KM**: Dissection of Androgen Receptor Signaling: Reconsideration of Direct Anabolic Action in Mature Bone. (Abstract#T202) J Bone Miner Res, 2007
- Semirale A, Zhang X-W, Wiren KM: DHT Treatment Reverses Gonadectomy-Induced Changes in Fat and Lean Mass in Male but Not Female Mice. (Abstract#T203) J Bone Miner Res, 2007
- *****Semirale A, Wiren KM: Therapeutic Considerations Regarding Androgen Administration. Sun Valley Workshop on Skeletal Tissue Biology, 2007
  ***Awarded Alice L. Jee Memorial Young Investigator Award**

Reportable Outcomes

Two peer-reviewed publications (with one more submitted and two in preparation) and three abstracts were published in 2006-2007 characterizing androgen action in the skeleton. In the work summarized here, we have characterized the second AR transgenic family set (AR2.3) with skeletally-targeted AR overexpression at 2 months of age. In contrast to the original AR3.6-transgenic family, the AR2.3-transgenic family demonstrates overexpression that is limited to mature osteoblasts and mineralizing osteocytes. *In vitro* analyses support findings established in the *in vivo* models.

Conclusions

These results have provided new insight into the importance of androgen action, through distinct AR transactivation, in mediating bone quality and changes that underlie envelope-specific responses. Our investigations of the mouse skeleton reveal that androgen signaling in immature osteoblasts and periosteal cells increases bone formation at the periosteal envelope (particularly in calvaria) and also influences processes that determine body composition and whole bone strength in AR3.6-transgenic mice. In contrast, analysis from AR2.3-transgenic mice suggests that signaling in mature osteoblasts/osteocytes primarily mediates the effects of androgens on matrix quality and/or mineralization with a negative impact on osteoblast vigor, and at least partially influences the effects of androgens to reduce bone turnover. A similar relationship may also exist in the human skeleton. These results indicate that mature osteoblasts are important mediators of androgen action to influence matrix quality and influence turnover parameters, but immature osteoblast lineage and periosteal cells are the major contributors to envelope-specific alterations in bone formation and stromal lineage commitment can be influenced by AR activation. As presented in Fig. 7 in our new model for androgen action, the effects of androgen are compartment specific. In trabecular bone, androgens reduce bone turnover but increase trabecular volume through an increase in trabecular number. In cortical bone, androgens inhibit osteogenesis at endosteal surfaces but increase bone formation at periosteal sites (12), to maintain cortical thickness yet displace bone further away from the neutral axis in males. Androgens also positively influence bone at intramembranous sites (12, 21). These results raise concerns that with increasing androgen action (as for example associated with anabolic steroid abuse) in young animals with still-growing skeletons, the bone matrix material is likely to be more stiff, less ductile and more damageable, and suggest that young anabolic steroid abusers may be at higher risk of stress fractures. Importantly, supplemental levels of androgen in eugonadal adults are also detrimental. In contrast, analyses in the mature adult indicate that androgen may be useful to treat reductions in bone mineral in hypogonadal males.

In addition, androgen administration increases muscle mass, partially mediated by effects on mesenchymal stem cell lineage commitment (22), likely to indirectly influence bone density through biomechanical linkage. But as we have shown, improvement body composition after androgen replacement is observed only in hypogonadal males. At the same concentration and for the same duration, hypogonadal females do not demonstrate the same improvement with androgen replacement. Supplemental androgen in eugonadal adults tends to worsen, not improve, body compositional changes that occur with aging.

Some of the negative consequences of AR overexpression in mature osteoblasts we have observed *in vivo* are consistent with our *in vitro* analyses. For example, there are reports, some in clonal osteoblastic cell lines, of effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation mediated by AR signaling (36-38). These findings are variable however, with other reports of no effect or even inhibition of osteoblast markers (39-41), consistent with our gene expression analysis in AR-transgenic mice. In addition, the effect of androgens on osteoblast proliferation is controversial. We have previously demonstrated that either stimulation or inhibition of osteoblast viability by androgen can be observed, and these effects are dependent on the length of treatment. Transient administration of nonaromatizable DHT can enhance transcription factor activation and osteoblast proliferation, while chronic treatment inhibits both mitogenic signaling and MAP kinase activity (42). Chronic DHT treatment *in vitro* can also enhance osteoblast apoptosis (14). Thus, these *in vitro* reports are consistent with the detrimental changes in matrix quality and osteoblast vigor we observe in the AR-transgenic model *in vivo*.

In summary, one of the effects of enhanced androgen signaling is altered whole bone quality and susceptibility to damage in the young, which may be revealed under extreme physical conditioning such as that experienced during military training. Our data also indicate a concern with androgen supplementation in eugonadal adults, both male and female. Finally, it does appear that androgen may be effective in improving body composition, including increased lean and reduced fat mass, but only in hypogonadal males with reduced sensitivity to the improvement in females. Combined, these results support the central hypothesis of distinct androgen signaling throughout osteoblast differentiation. Androgen action in the skeleton is complex and will likely not provide for improved skeletal dynamics in the still-growing skeleton or eugonadal adult, although the effect in the hypogonadal male is more positive.

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The Effect of Oxandrolone Treatment on Human Osteoblastic Cells

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Objective: Oxandrolone, administered to severely burned children over the first year postburn, produces increased lean body mass by 6 months; however, an increase in total body bone mineral requires 12 months. Consequently, this bone mineral response may be due to increased muscle mass. Alternatively, oxandrolone may act directly on bone. The current study seeks to determine whether oxandrolone can transactivate the androgen receptor in osteoblasts. **Methods:** Collagen, alkaline phosphatase, osteocalcin, osteoprotegerin, and androgen receptor abundance were determined by qRT-PCR, confocal laser scanning microscopy, or immunoquantitative assay. To determine the effect of oxandrolone on gene expression in differentiated cells, osteocytic cultures were grown to confluence in differentiation medium and then treated 24 hours or 5 days with 15 μg/mL oxandrolone. **Results:** Increased nuclear fluorescence of the androgen receptor and increased cellular type I collagen were observed with oxandrolone at 15 and 30 μg/mL but not at lower doses. Alkaline phosphatase (7%–20%) and osteocalcin (13%–18%) increases were modest but significant. Short-term treatment produced no significant effects, but at 5 days androgen receptor levels were increased while collagen levels were significantly decreased, with little effect on alkaline phosphatase, osteocalcin, or osteoprotegerin. **Conclusions:** These data suggest oxandrolone can stimulate production of osteoblast differentiation markers in proliferating osteoblastic cells, most likely through the androgen receptor; however, with longer treatment in mature cells, oxandrolone decreases collagen expression. Thus it is possible that oxandrolone given to burned children acts directly on immature osteoblasts to stimulate collagen production, but also may have positive effects to increase bone mineral through other mechanisms.

Long-term use of the orally administered anabolic agent oxandrolone has been shown to increase both lean body mass and bone mineral content in severely burned children when

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given over the first year postburn.[1] Oxandrolone and recombinant human growth hormone[2] were shown to be effective in recovering bone that would ordinarily be lost following the burn injury, resulting in markedly reduced bone formation[3] hypocellularity at the mineralization front of bone[3,4] and decreased marrow stromal cell differentiation into osteoblasts.[4]

Increased endogenous glucocorticoid production is likely responsible for the acute bone loss observed in severely burned patients.[3,4] Notably, with both oxandrolone and recombinant human growth hormone, an increase in lean body mass precedes an increase in bone mineral content by 3 to 6 months.[1,2] Thus it is not clear whether these anabolic agents increase bone mineral content secondary to increased muscle mass and hence increased skeletal loading, or whether they act directly on osteoblastic cells. Oxandrolone is an anabolic steroid with the ability to transactivate the androgen receptor (AR), which may be one mechanism underlying the anabolic response to therapy. The aim of our study is to determine whether oxandrolone can increase osteoblastic production of type I collagen and whether this action is mediated by AR signaling.

MATERIALS AND METHODS

Human osteoblast cell cultures

Freshly discarded human cancellous bones were obtained from healthy young patients undergoing osteotomy. The bone fragments were washed with serum-free α–minimum essential medium (α-MEM, Flow Laboratories, McLean, VA). Then, the fragments were digested in the medium with 1 mg/mL collagenase for 2 hours at 37°C. The enzymatic reaction was stopped by adding an equal volume of α-MEM with 10% fetal bovine serum (FBS). The supernatant containing the released cells was recovered. Washing and recovering were repeated 3 times. The cells were transferred to a centrifuge tube and centrifuge 10 minutes at $100 \times g$ to harvest the cells. The cell pellet was resuspended in 5 mL of fresh medium. The single cell suspension was cultured in α-MEM containing antibiotics [penicillin (100 U/mL), streptomycin sulphate (100 μg/mL)] and 10% FBS in a humidified incubator at 37°C under an atmosphere of 5% CO_2 and 95% air. After confluence, adherent cells were collected by trypsinizing with 0.025% trypsin-EDTA and resuspended in α-MEM. Only passages 4 to 8 were used in this study. Medium was changed every other day.[4]

Osteocytic cell culture

Osteoblasts in vitro progress through several developmental stages that correlate with osteoblast development in vivo: from committed preosteoblasts to mature, differentiated osteoblasts, and finally to osteocyte-like cells embedded in mineralized extracellular matrix.[5–7] To characterize the effects of oxandrolone in osteocytic cells, human osteoblast cells were cultured in α-MEM with 5% fetal bovine serum containing antibiotics for 10 days until confluence, then switched to BGJ_b medium containing 50 μg/mL ascorbic acid and 3 mM β-glycerol phosphate.[7] Cultures were maintained in differentiation medium for 7 days for mature osteoblast/osteocyte development,[7] then treated with oxandrolone for time course and dose-response analysis. These highly confluent osteocytic cultures were treated in 5% charcoal-stripped serum with either 15 μg/mL of oxandrolone for 24 hours or 5 days, or

with increasing concentrations of oxandrolone (0, 1, 5, 10, and 15 μg/mL) for 5 days. Total RNA was isolated for gene expression analysis as described below.

Immunohistochemistry for collagen type I and AR

Immunohistochemical staining was carried out for both the AR[8,9] and type I collagen. Human osteoblastic cells were trypsinized, seeded on slide chambers (Lab-Tek, Nalge Nunc International, Rochester, NY) at a concentration of 1×10^5cells/mL and cultured for 3 days until they achieved 60% to 70% confluence. Oxandrolone was used to stimulate the cells at the concentration of 1, 5, 10, 15, and 30 μg/mL for 24 hours. Stock solution containing 2.5 mg diluted in 2 mL of DMSO was prepared and subsequently diluted in culture media, while control nonstimulated cells received the same solution without oxandrolone. Thereafter, the medium was discarded, the cells were washed with phosphate buffered saline (PBS), and fixed in -20°C methanol/acetone (50:50) for 20 minutes and permeabilized with 0.5% Triton X-100 (Sigma, St Louis, MO). The polyclonal antibody used for collagen I was applied overnight at the dilution of 1:200 (Research Diagnostics Inc, Flanders, NJ). The polyclonal antibody for AR (Neomarkers, Fremont, CA) was used at the dilution of 1:30. The next day, FITC-conjugated goat anti-rabbit IgG was used as secondary antibody (Neomarkers) and cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Vector Mounting Medium, Burlingame, CA). Images were captured using the laser confocal scanning microscope (Zeiss LSM 510, Jena, Germany) with a 63$\times$ objective. Optical sections were obtained from the cells by capturing single images of central cell focal planes. Three microscopic fields were captured for the control group of each cell line and the same was done for cells treated with oxandrolone. To proceed to an analysis of AR immunohistochemistry, the intensity of fluorescence was measured in the nuclei (AR) in 2 cells per 3 fields, using Image Tool software (University of Texas Health Science Center, San Antonio).

Assay of alkaline phosphatase

Human osteoblastic cells were cultured in α-MEM containing antibiotics and 10% FBS. The cells were challenged with increasing concnetrations of oxandrolone (0, 1, 5 10, 15 μg/mL). After 24 hours of treatment, the levels of alkaline phosphatase activity were determined using a commercial kit (Pierce Biotechnology, Inc, Rockford, IL). The cells were washed with cold PBS and subjected to 3 freeze-thaw cycles. These samples were assayed for enzymatic activity with *p*-nitrophenyl phosphate as a substrate. Sample absorbance was measured at 400 nm with microplate reader. Results were expressed in ALP (OD)/protein (OD).

Immunoquantitative assay for collagen type I and osteocalcin

To perform the immunoquantitative assay for type I collagen[4] and osteocalcin, human osteoblastic cells were subcultured on 96-well plates until they achieved 70% confluence. Oxandrolone was used to stimulate the cells at the concentration of 0, 1, 10, 15, and 30 μg/mL for 24 hours. Cells were harvested and washed with cold PBS, fixed in methanol for 10 minutes at -20°C and washed with PBS. They were then preincubated with primary antibody for type I collagen and osteocalcin (Santa Cruz Biotechnology Inc, CA), respectively, overnight at 4°C, followed by secondary biotinylated IgG for 30 minutes at room temperature. After washing with PBS, cells were exposed to *p*-nitrophenyl phosphate containing levamisole to inhibit the generation of *p*-nitrophenol by endogenous alkaline phosphatase

for 8 minutes. Sample absorbance was measured at 400 nm with an ELISA reader. The values (OD) were normalized with protein concentration (OD).

Real-time quantitative reverse transcription–polymerase chain reaction (qRT-PCR) on cultured human osteocytic cells

Total RNA was isolated from osteocytic cultures and gene expression characterized by qRT-PCR analysis using human primers from Qiagen (Valencia, CA) specific for AR and the osteoblast marker proteins alkaline phosphatase, type I collagen, osteocalcin, and osteoprotegerin. The qRT-PCR analysis was performed with the iCycler IQ Real Time PCR detection system (Bio-Rad Laboratories, Inc, Hercules, CA) using a one-step QuantiTect SYBR Green RT-PCR kit (Qiagen) on DNase-treated total RNA. Relative expression of the RT-PCR product was determined using the comparative $\Delta\Delta C_t$ method after normalizing expression with fluorescence to the specific RNA-binding dye RiboGreen (Molecular Probes, Eugene, OR) as previously described.[10] Real-time qRT-PCR efficiency was determined for each primer set using a fivefold dilution series of total RNA and did not differ significantly from 100%. Individual reaction kinetics were also analyzed to ensure each real-time RT-PCR did not differ significantly from 100% efficiency. Following PCR, specificity of the PCR reaction was confirmed with inverse derivative melt curve analysis. Data is presented as mean $\pm$ SEM.

Statistical analyses

Data are reported as mean $\pm$ SEM. The statistical significance of intergroup differences was tested using the Student *t* test when the variances were equal. *P* less than .05 was considered significant.

RESULTS

Oxandrolone treatment in proliferating human osteoblastic cells

Proliferating cultures of normal human osteoblastic cells were treated for 24 hours with 30 μg/mL oxandrolone or vehicle to determine the acute response to oxandrolone treatment. Although the affinity for oxandrolone for AR is approximately 100-fold lower than testosterone, oxandrolone treatment still results in AR transactivation.[11] To characterize the ability of oxandrolone to stimulate translocation of the AR to the nucleus, confocal laser scanning microscopy was employed. When control cultures were stained for AR, diffuse cytoplasm staining but only weak nuclear staining were observed on osteoblasts nonstimulated with oxandrolone (Fig 1a). After stimulation with oxandrolone, increased fluorescence of AR in the nuclei was seen (Fig 1b), suggesting transactivation by oxandrolone at these concentrations.

We next assessed the effect of oxandrolone treatment on type I collagen, the major constituent of the bone matrix. Immunohistochemistry for collagen type I on cultured osteoblast cells demonstrated an increase in collagen that was dose dependent. When compared to control group (Fig 1c), a significant increased intensity of fluorescence was seen for cells stimulated with oxandrolone. This was observed when the concentration of oxandrolone was 15 μg/mL (Fig 1f) and 30 μg/mL (Fig 1d).

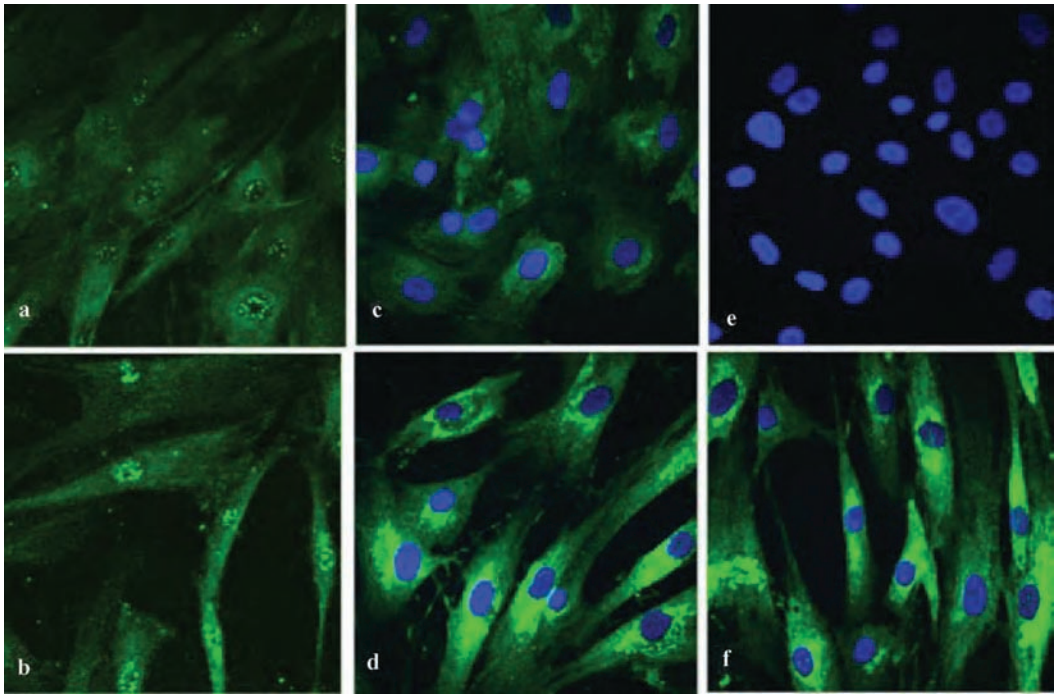

Figure 1. Confocal scanning laser microscopic analysis of human osteoblastic cells after oxandrolone treatment. (a) Confocal scanning laser microscopy depicting AR nuclear fluorescence of human osteoblastic cells not stimulated with oxandrolone, (b) confocal scanning laser microscopy depicting the increased AR nuclear fluorescence of human osteoblastic cells stimulated with oxandrolone 30 μg/mL, (c) confocal scanning laser microscopy depicting cytoplasmic fluorescence of type I collagen in human osteoblastic cells not stimulated with oxandrolone, (d) confocal scanning laser microscopy depicting increased cytoplasmic fluorescence of type I collagen in human osteoblastic cells stimulated with oxandrolone 30 μg/mL, (e) internal negative control for primary antibodies of AR and type I collagen, (f) confocal scanning laser microscopy depicting increased cytoplasmic fluorescence of type I collagen in human osteoblastic cells stimulated with oxandrolone 15 μg/mL. Compare to Figures 1c and 1d.

To determine the effect of oxandrolone on additional markers of osteoblast activity, osteoblastic cultures were treated with or without oxandrolone. Alkaline phosphatase activity was determined in cells treated with oxandrolone for 24 hours (Fig 2), and there was an increase (7%–20%) in activity in a dose-dependent manner. The differences were statistically significant ($P < .05$) but the changes were minor compared to the control group. We also performed immunoquantitative assays for type I collagen and osteocalcin on osteoblastic cells. In cultures treated with oxandrolone (10 or 15 μg/mL), collagen was increased 21% to 35% (Fig 3; $P < .05$). There were no differences in the groups treated with low concentrations of oxandrolone (1 or 5 μg/mL) compared to control group. Thus, after multiple repetitions of the experiment, the data confirmed the results observed using immunofluorescence.

We next assessed the levels of osteocalcin, an important biochemical indicator of bone turnover.[12] Cells treated with oxandrolone of 10, 15, and 30 μg/mL produced a greater level of osteocalcin (11%–18%) compared to the control group. The differences were statistically

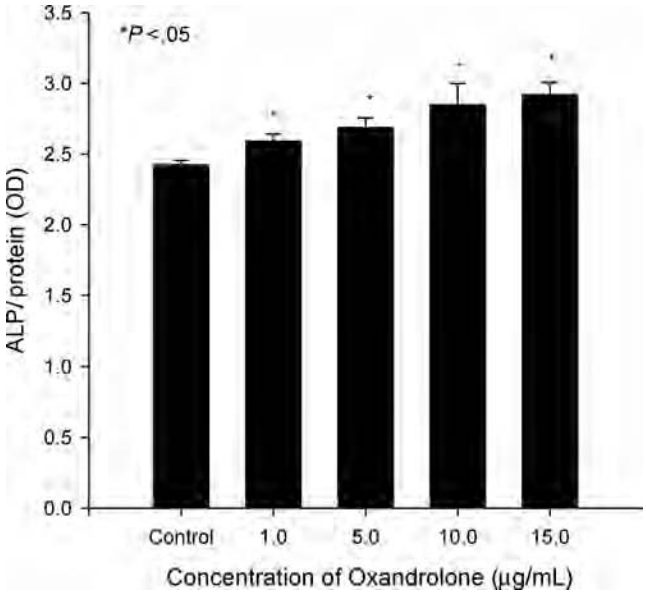

Figure 2. Stimulation of alkaline phosphatase activity by oxandrolone in proliferating human osteoblastic cultures. Proliferating cultures were treated acutely for 24 hours with 0, 1, 5, 10, and 15 μg/mL oxandrolone. Data are expressed as mean $\pm$ SEM of 6 determinations.

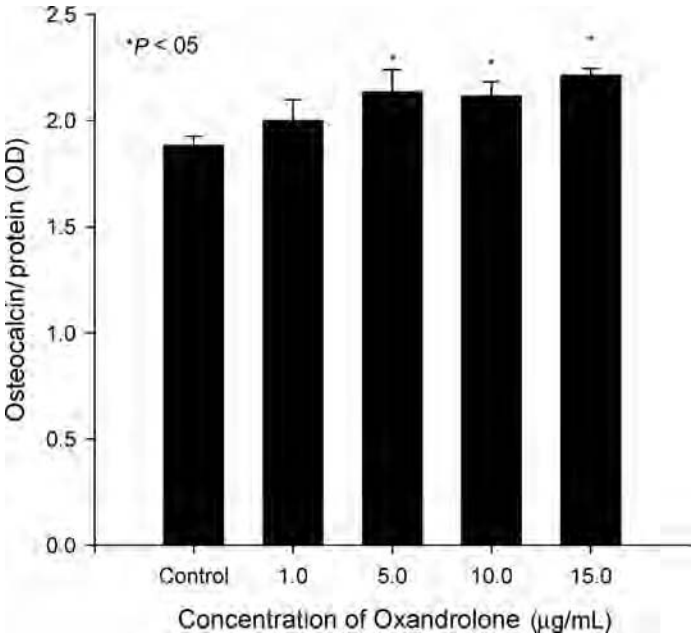

Figure 3. Increased osteocalcin levels after oxandrolone treatment. Human osteoblastic cultures were exposed to oxandrolone (0, 1, 5, 10, and 15 μg/mL) for 24 hours. Data are expressed as mean $\pm$ SEM of 6 determinations.

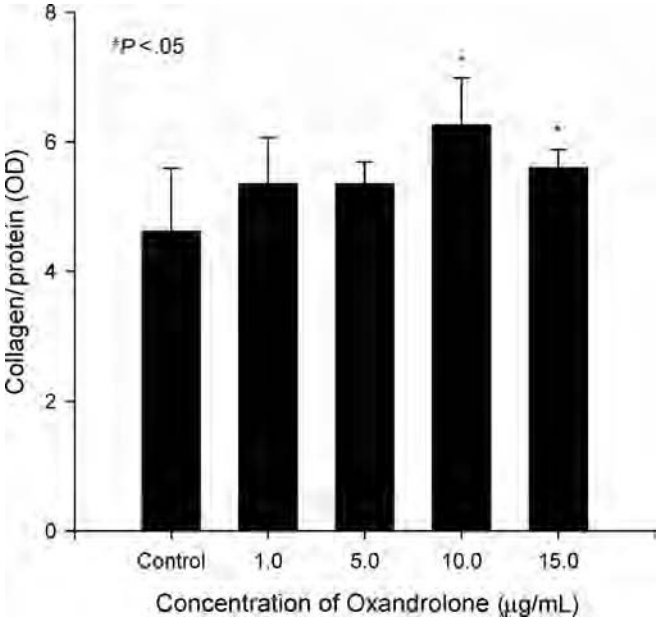

Figure 4. Enhanced type I collagen secretion after oxandrolone exposure. Proliferating cultures were treated with 0, 1, 5, 10, and 15 μg/mL oxandrolone for 24 hours. Data are expressed as mean ± SEM of 6 determinations.

significant (Fig 4; $P < 0.05$) but again the increase was small. Thus, the production of osteocalcin mirrored the pattern of alkaline phosphatase activity.

AR concentrations increase as osteoblasts differentiate, reaching their highest levels in osteocytic cultures.[13] Furthermore, the most abundant cell in bone is the osteocytic cell. We therefore determined the effect of oxandrolone treatment on gene expression in mature osteocytic cultures. Normal human osteoblasts were cultured for 10 days, then switched to differentiation medium containing ascorbic acid and β-glycerol phosphate. After 7 days, osteocytic cells were treated with 15 μg/mL oxandrolone. Total RNA was isolated after 24 hours or 5 days treatment, and gene expression characterized by qRT-PCR analysis using human primers specific for AR, type I collagen, alkaline phosphatase, osteocalcin, and osteoprotegerin (Fig 5). Although there was little effect of oxandrolone after 24 hours of treatment in osteocytic cells on type I collagen, alkaline phosphatase, osteocalcin, or osteoprotegerin mRNA abundance, a nonsignificant increase in AR mRNA was noted. This is consistent with other reports documenting an increase in AR after androgen treatment in osteoblasts.[14] After stimulation with oxandrolone for 5 days, a significant decrease in expression of type I collagen was seen ($P < 0.01$). Thus, longer treatments with oxandrolone in osteocytic cultures reduced collagen expression. These data are consistent with dose-response studies performed after 5 days of treatment with increasing concentrations of oxandrolone (Fig 6), where AR mRNA is modestly increased but collagen levels are significantly decreased in a dose-dependent fashion with oxandrolone treatment.

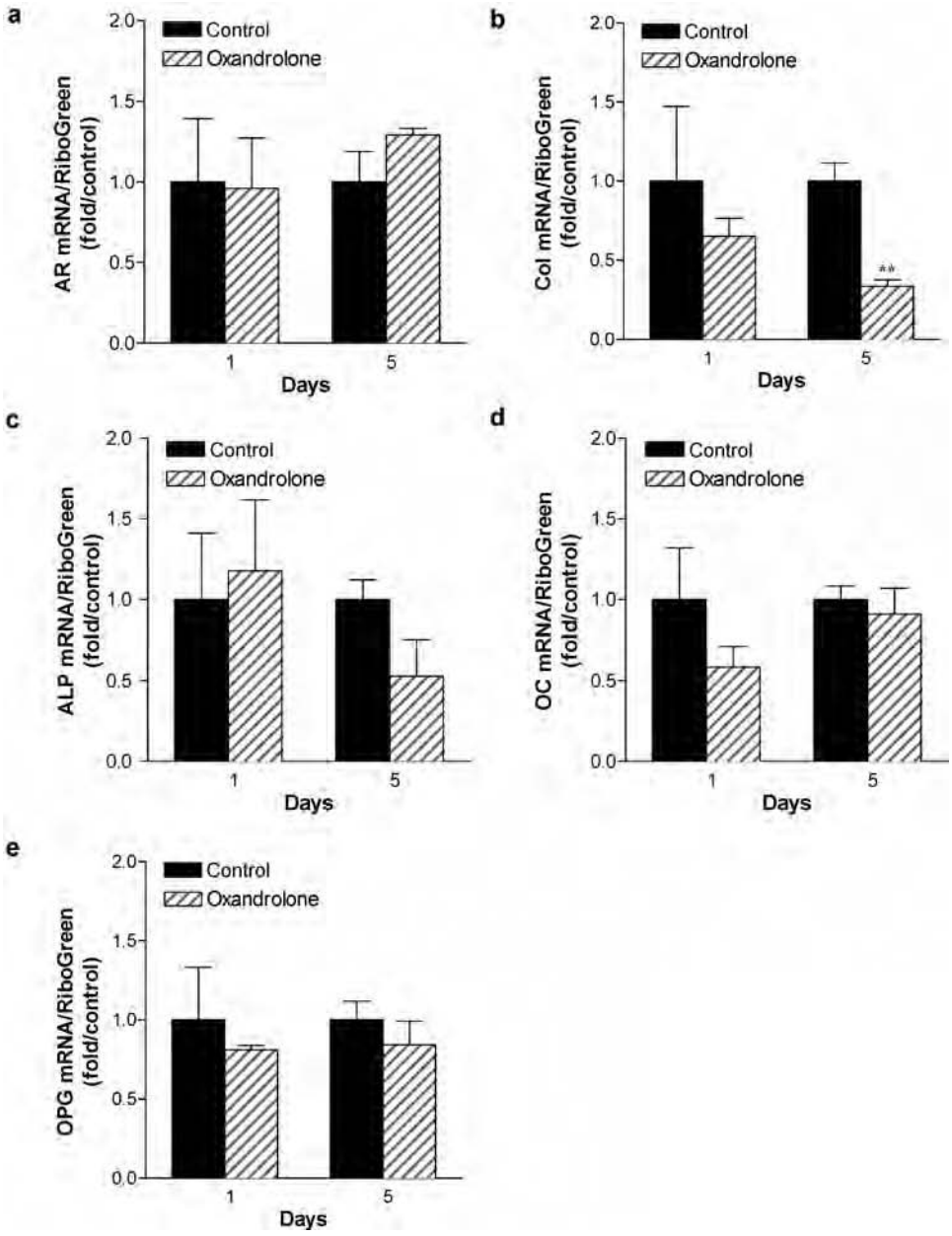

Figure 5. Consequence of oxandrolone treatment on gene expression in normal human osteocytes: time course analysis. Normal human osteoblastic cells were cultured for 10 days to confluence, then switched to differentiation medium containing ascorbic acid and β-glycerol phosphate. After 7 days, osteocytic cells were treated with 15 μg/mL oxandrolone. Total RNA was isolated after 24 hours or 5 days treatment, and gene expression characterized by qRT-PCR analysis using human primers specific for AR, type I collagen (col), alkaline phosphatase (ALP), and osteocalcin (OC) and osteoprotegerin (OPG). $n = 3$ to 4. $^{**}P < .01$.

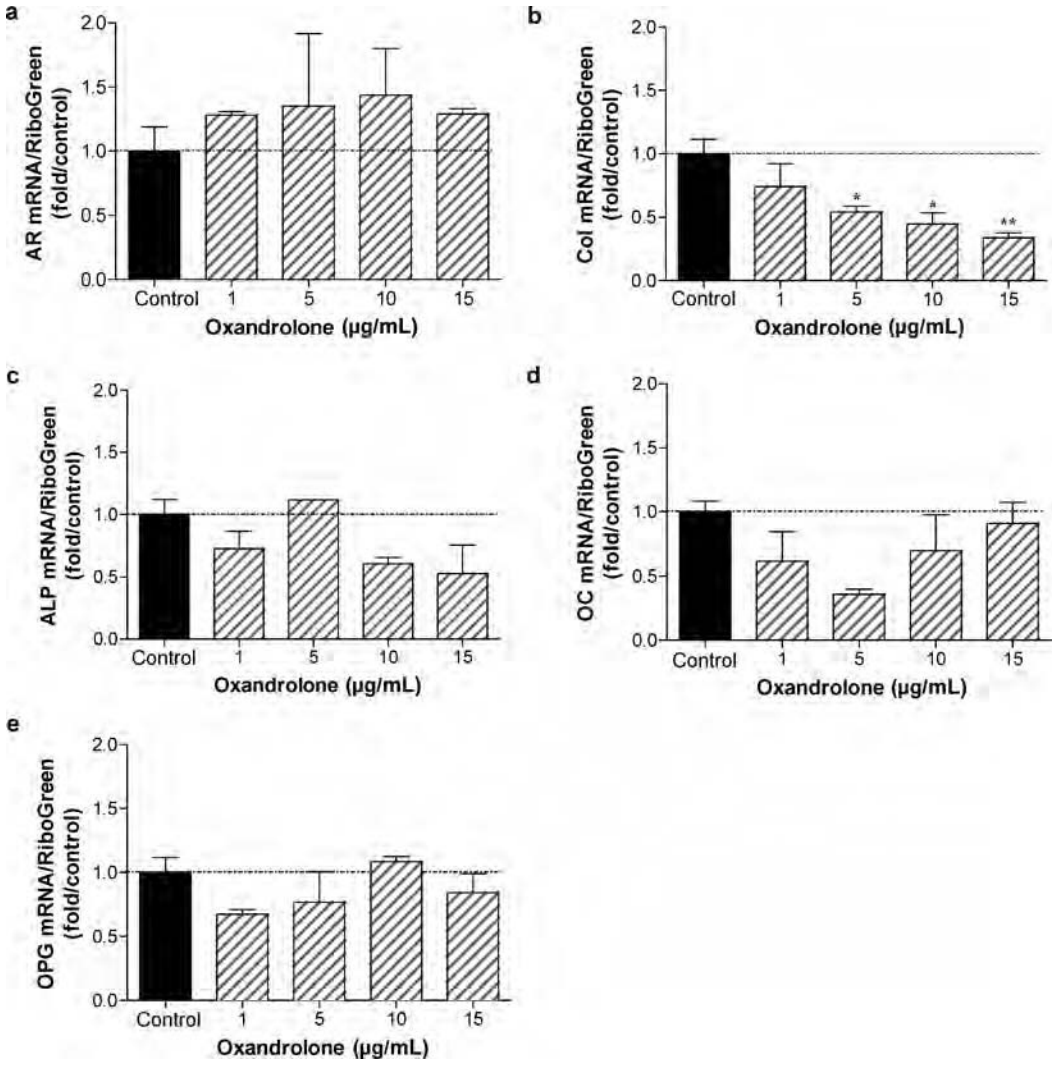

Figure 6. Consequence of oxandrolone treatment on gene expression in normal human osteocytes: dose-response analysis. Normal human osteoblastic cells were cultured as described in Figure 5. Osteocytic cultures were treated for 5 days with 0, 1, 5, 10, and 15 μg/mL oxandrolone. Total RNA was isolated and gene expression characterized by qRT-PCR analysis using human primers specific for AR, type I collagen (col), alkaline phosphatase (ALP), osteocalcin (OC) and osteoprotegerin (OPG). $n = 2$ to 4. $^{*}P < .05$; $^{**}P < .01$.

DISCUSSION

In this study we have shown that stimulation of cultured human osteoblasts by oxandrolone results in immunofluorescent detection of AR in the nucleus and increased osteomarkers in these osteoblasts. These data suggest that oxandrolone directly targets human osteoblasts by means of the AR, resulting in increased expression of osteoblast differentiation markers after short-term treatment. Therefore, oxandrolone may act directly on the osteoblast in addition to effects that result in increasing skeletal loading.

Immunohistochemistry and confocal laser scanning microscopy (CLSM) were used to evaluate the expression of AR and type I collagen in osteoblasts, with several advantages over regular fluorescence microscopy.[15,16] While in conventional wide-field fluorescence microscopy the emitted light coming from regions above and below the focal plane is collected by the objective lens and contributes to an out-of-focus blur in the final image, in the CLSM a diaphragm rejects the out-of-focus information.[15,16] Therefore, tissue thickness does not interfere with the resulting fluorescence. The increased expression of type I collagen by osteoblasts was also determined by immunoassay confirming the results observed with immunohistochemistry.

The AR is a steroid receptor that generally mediates biologic responses to androgens. The increased expression of AR in the present study is consistent with that documented in the literature.[14] In bone tissue the AR is expressed in a variety of cell types, but its specific role in the maintenance of skeletal homeostasis remains controversial.[17] In an experimental study, androgen deficiency was shown to result in a substantial loss of cancellous bone in the axial and appendicular skeleton of aged male rats and that this osteopenia is associated with a sustained increase in bone turnover.[18] Consistent with this, the bone phenotype that develops in a global AR null (ARKO) male mouse model is a high-turnover osteopenia, with reduced trabecular bone volume, and a significant stimulatory effect on osteoclast function.[19,20]

Alkaline phosphatase is well recognized as a marker that reflects osteoblastic activity.[21] Kasperk et al reported that androgens increase the alkaline phosphatase activity in osteoblast culture.[22–24] However, there are also reports of androgens either inhibiting[25] or having no effect on alkaline phosphatase activity,[26,27] which may reflect both the complexity of osteoblastic differentiation and the various model systems employed. In a clinical study, Murphy et al[1] have shown that oxandrolone administration increases levels of serum alkaline phosphatase in treated patients by 6 months versus controls. In the present study, the cells treated with oxandrolone produced a greater level of alkaline phosphatase. The elevated activity of this cellular enzyme suggests an early increase in the osteoblast differentiation process.

Osteocalcin appears to be a bone-specific gla-containing protein, accounting for 10% to 20% of the noncollagenous protein in bone. While the in vivo function of osteocalcin remains unclear, its affinity for bone mineral constituents implies a role in bone formation. Hence it has been shown that osteocalcin is a biochemical indicator of bone turnover.[12] In our study, proliferating cells treated for 24 hours with oxandrolone produced small but statistically significant increases in osteocalcin production compared to control group.

To test the effect of oxandrolone on osteocyte-like cells differentiated from osteoblasts at the molecular level, the human osteoblastic cells were cultured in a differentiation medium and then exposed to oxandrolone. The results showed that short-term treatment produced little effect on type I collagen, alkaline phosphatase, osteocalcin, osteoprotegerin, or AR mRNA. Long-term treatment decreased type I collagen expression, consistent with decreased collagen expression observed by Wiren et al[16] in AR-transgenic mice with targeted AR overexpression in the osteoblast lineage.[16] The results described here are consistent with observations reported in vivo with male AR-transgenic mice, where calvarial thickening is observed and cortical formation is altered with surface periosteal expansion but inhibition of inner endosteal deposition, consistent with the known effects of androgen to stimulate periosteal apposition. The dramatic inhibition at the endosteal envelope may be

responsible for a modest decrease in cortical bone area and reductions in biomechanical properties observed.[16] Thus, taking all data together, our results suggest that osteoblast cells are targeted by androgens to transactivate AR in bone.

Murphy et al[1] have recently shown that oxandrolone administration increases lean body mass 3 to 6 months before an increase in bone mineral content is observed. On the other hand, we have found an increase in collagen production when high doses of oxandrolone were used to stimulate osteoblasts. The applicability of our findings in this study to the in vivo effects of oxandrolone in burned children[1] is not clear. While an in vitro environment may not adequately reproduce the in vivo situation, many potential variables are eliminated during in vitro studies, and it is possible to investigate a specific effect of a drug on cells. The time frame of in vivo and in vitro settings is plainly different, and the concentration of oxandrolone used to stimulate cells in the in vitro setting is probably higher than the one used in the clinical trial.[1] Therefore, the results may not be strictly comparable. Although this study demonstrates that oxandrolone is capable of affecting the osteoblast AR and stimulating type I collagen synthesis, regulatory mechanisms that are present only in a complex in vivo situation may account for increased collagen turnover or degradation after synthesis.

Moreover, the delay in the increase of total body bone mineral content reported in the clinical study by Murphy et al[1] may be explained in part by the acute inhibition of bone formation and osteoblast differentiation after a severe burn as previously reported.[3,4] Thus there may have been a lack of osteoblasts, and therefore osteoblast AR, to mediate a response to oxandrolone.

In conclusion, the use of high-dose oxandrolone results in increased nuclear fluorescence of the osteoblast AR, increased osteoblast differentiation markers in cultured osteoblasts and decreased bone marker in cultured osteocyte-like cells in vitro. Oxandrolone may have the ability to directly stimulate bone collagen synthesis, over and above its effect on skeletal loading effected through an increase in lean body mass following long-term treatment. However, longer exposure may no longer be directly anabolic in mature bone cells. This observation may help to explain the variability and confusion regarding androgen actions on the skeleton.

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Androgens are known to have pervasive effects on target tissues including muscle and fat, yet the effects on bone remain poorly characterized. To gain insight into the cell types important for mediating androgen action, we constructed and compared two distinct transgenic lines of mice employing different α_1(I)-collagen promoter fragments to control skeletally-targeted androgen receptor (AR) overexpression. The col3.6 AR-transgenic (AR3.6-tg) mice demonstrate AR overexpression throughout the osteoblast lineage including bone marrow stromal cells (BMSC), while in col2.3 (AR2.3-tg) mice AR overexpression is restricted to mature osteoblasts and osteocytes. Complex skeletal analysis using morphological characterization by µCT, dynamic and static histomorphometric analysis, dual-energy x-ray absorptiometry (DXA), biomechanical testing and gene expression studies all indicate that androgen signaling in mature osteoblasts during growth produces a low turnover state, the consequences of which are detrimental to overall matrix quality and bone strength. Unexpectedly, analysis of AR3.6-tg mice also revealed body composition changes in adult males, including reduced fat mass and increased lean mass, an effect not seen in females or in AR2.3-tg mice.

To further examine the effects of enhanced AR signaling on body composition and skeletal quality, an experimental paradigm of protracted hormone ablation followed by steroid replacement was employed. Control (B6D2F2) and AR-tg mice were sham operated or gonadectomized at 3 months of age and the effect of nonaromatizable dihydrotestosterone (DHT) was determined after an 8 week delay, allowing for gonadectomy-induced changes to develop. Following 6 weeks of treatment, the effects of androgen on bone and whole body composition was assessed by DXA. In wild type females, ovariectomy (OVX) resulted in a 13.8 % decrease in BMD while BMC fell 22 % compared to sham (n=14-17). Systemic DHT administration significantly improved BMD and BMC compared to placebo-treated animals (n=14-26). Orchidectomy (ORX) also resulted in reduced BMD and BMC in males (10.5% and 20.3%, respectively)

and 6 weeks of DHT treatment was sufficient to improve or fully restore these deficits in bone mineral. Gonadectomized AR-tg mice also lost bone mineral.

In AR2.3-tg females, BMD and BMC decreased 22.2% and 24.1%, respectively. In contrast to littermate controls, DHT treatment in AR2.3-tg female mice did not improve either bone measure when compared to placebo (n = 3-14). Albeit a small cohort, preliminary results indicate that AR2.3-tg males do not significantly lose BMD or BMC following ORX (n=3-5).

In AR3.6-tg male mice BMD decreased 5.1% while a 15.1 % loss in BMC was observed after ORX.

Steroid loss associated with ageing is known to influence body composition. In addition, in rodent models, gonadectomy has been shown to reduce lean mass and increase fat as a percent of body weight. Consistent with this, our results show that in control males (n = 8-11), lean mass as a percent of body weight was significantly reduced (7.7 %, $p < 0.001$) following ORX while conversely, the percent fat mass increased (7.6 %, $p < 0.001$) compared to sham controls. DHT treatment was beneficial, improving or fully restoring body composition changes induced by ORX compared to placebo. In females (n = 4-10) a similar trend was observed with increased fat (2.2 %) and reduced lean mass (2.3 %) after OVX. Surprisingly, females were resistant to the effects of DHT to restore body composition.

AR2.3-tg mice demonstrated changes in body composition after gonadectomy that mirrored wild types (n=3-9). Again similar to wild-type controls, DHT improved ORX-induced alterations in % fat and % lean mass in AR2.3-tg males but not females.

In contrast to gonadectomized models, DHT treatment in intact wild type mice had a negative impact on bone mineral and body composition in both males and females. Males (n = 14-23) lost 4.4 % lean mass ($p < 0.05$) and increased fat mass by 4.3 % ($p < 0.05$) compared to placebo, with similar but less dramatic changes observed in females (n = 14-26). These results show both males and females increase fat and lose lean mass following gonadectomy. In the bone-targeted AR-overexpression model, enhanced androgen signaling does not influence the response to DHT suggesting that circulating factors derived from bone likely do not play a role in the body composition response to androgen therapy.

Taken together these results indicate that improvements in bone mass with androgen treatment after gonadectomy in wild-type mice are likely mediated through effects on extra-skeletal tissues, not osteoblasts. Consistent with

detrimental effects of enhanced androgen signaling on bone quality, DHT treatment in intact controls significantly reduced bone mass in both males and females. These findings demonstrate that after a sustained hypogonadal period, androgen effectively treats bone loss but that enhanced androgen signaling directly in bone is not anabolic in either male or female adults. The data suggests that targeting androgen response to mature osteoblasts is not beneficial for bone formation, and raise concerns regarding androgen administration or anabolic steroid abuse in healthy individuals in both sexes.

These findings suggest androgen administration has therapeutic advantages in the hypogonadal, but should be approached with caution in healthy adults.

Targeting of androgen receptor in bone reveals a lack of androgen anabolic action and inhibition of osteogenesis.

A model for compartment-specific androgen action in the skeleton.

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Running Title: Inhibition Of Bone Formation By Androgen

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MICROABSTRACT

The consequence of enhanced androgen signaling in mature osteoblasts was investigated in AR2.3-transgenic mice. Skeletal analyses show low turnover and inhibition of osteogenesis at the endosteum. These changes are detrimental to overall matrix quality, bone fragility and whole bone strength.

ABSTRACT

Introduction: Androgens are anabolic hormones that affect many tissues, including bone. However, a positive effect of androgen treatment on bone in eugonadal subjects has not been observed and clinical trials have been disappointing. The androgen receptor (AR) mediates biological responses to androgens. In bone tissue, both AR and the estrogen receptor (ER) are expressed. Since androgens can be converted into estrogen, the specific role of the AR in maintenance of skeletal homoeostasis remains controversial.

Methods: The goal of this study was to use skeletally targeted overexpression of AR in differentiated osteoblasts as a means of elucidating the specific role(s) for AR transactivation in the mature bone compartment. AR was cloned downstream of a 2.3-kb α1(I)-collagen promoter fragment and used to create AR2.3-transgenic mice.

Results: AR2.3-transgenic mice demonstrate no difference in body composition, testosterone, or 17ß-estradiol levels, but transgenic males have reduced serum osteocalcin and a bone phenotype is observed. In cortical bone, high-resolution micro-computed tomography reveals no difference in periosteal perimeter but a reduction in cortical bone area due to an enlarged marrow cavity. Medullary bone formation rate at the endosteal surface is significantly inhibited. Biomechanical analyses shows decreased whole bone strength and quality, with significant reductions in all parameters tested. Trabecular morphology is altered, with increased bone volume comprised of more trabeculae that are closer together but not thicker. With the exception of increased osteoprotegerin expression, levels of selected osteoblastic and osteoclastic genes in whole bone are reduced.

Conclusions: Enhanced androgen signaling directly in bone results in inhibition of bone formation by differentiated osteoblasts, with a phenotype reflecting low turnover. The

consequence of androgen action is compartment-specific; anabolic effects are exhibited exclusively at periosteal surfaces, but in mature osteoblasts androgens inhibit osteogenesis with detrimental effects on matrix quality, bone fragility and whole bone strength. These results indicate that direct androgen action in mature osteoblasts is not anabolic, and raise concerns regarding anabolic steroid abuse in the developing skeleton.

Key words: androgen receptor; osteocyte; μCT; biomechanics; dual energy X-ray absorptiometry

INTRODUCTION

Androgens are steroids that are generally characterized as anabolic hormones, with effects on many tissues including brain, the immune system, the cardiovasculature, muscle, adipose tissue, liver and bone. Given the large increase in drug sales for testosterone (the major androgen metabolite), estimated at over 500% in the last fifteen years, analysis of the biological consequences of androgen signaling should receive considerable research attention. However, the specific effects of androgen on the skeleton remain poorly characterized and understudied. Since osteoporosis, a low bone mass disease characterized by bone fragility, is often coupled with a hypogonadal state in both men and women, sex steroids are implicated in the maintenance of skeletal health. Although both estrogen and androgen circulate in men and women, albeit at different levels, the influence of each on the remodeling skeleton is distinct (1). Estrogens are thought to act to maintain adult bone mass predominantly through an inhibition of bone resorption by the osteoclast, i.e. as anti-resorptive agents, which protect the skeleton from further loss of bone. Nonaromatizable androgens such as 5α-dihydrotestosterone (DHT), on the other hand, have been proposed as possible bone anabolic agents (2,3).

In support of a positive effect of androgen action on the skeleton, free testosterone concentrations correlate with bone mineral density (BMD) in elderly men; testosterone levels also correlate with muscle mass and strength (4). Testosterone treatment is effective at ameliorating bone loss during aging, but only in men with low testosterone levels (5,6). Conversely, men undergoing androgen deprivation therapy for prostate cancer show significantly decreased BMD (7) and an increase in clinical fractures (8). Interestingly, combination therapy with both estrogen and androgen in postmenopausal women indicate an improved response compared to estrogen alone (9,10). During

growth, there are gender differences in skeletal morphology that develop with puberty particularly in cortical bone, with radial expansion that is predominantly observed in boys (11). Testosterone is also effective in stimulating bone turnover in boys with either growth or pubertal delay (12). Combined, these findings suggest that androgens serve important functions to both maintain bone mass in the adult, and in addition, to influence the growing, modeling skeleton (13).

Nevertheless, a controversy exists regarding the consequences and/or importance of androgen signaling on skeletal homeostasis. Whether the observed effects of circulating testosterone are due to direct effects on bone is complicated by the fact that androgens influence a variety of tissues, most importantly muscle, which is known to be associated with bone health. Nonetheless, bone is a direct target tissue with respect to androgen action. AR is expressed in the cell types required for skeletal formation and homeostasis, including mesenchymal stromal precursors (14), osteoblasts (15), osteocytes (16) and osteoclasts (17). An additional complication for interpretation of the direct effects of testosterone result from the consequences of its metabolism. Since testosterone serves as the substrate for estradiol synthesis through the action of the enzyme aromatase, systemic testosterone may have effects mediated predominantly or exclusively through activation of estrogen receptor (ER) signaling. Therefore, a specific role for AR signaling cannot be inferred with simple testosterone therapy.

In addition, not all of the studies examining the association of testosterone levels with BMD in adults have actually shown a positive correlation. In general, correlations between bone mass and serum androgen concentrations in adult men have been either weak or insignificant (18-20). Furthermore, many of the various clinical trials examining androgen therapy have not

demonstrated robust effects on bone, including treatment with anabolic steroids (21). As noted above, in most studies that do show an increase in BMD, the most marked improvement is observed only in men with the lowest testosterone levels (5). Notably, a positive effect of androgen treatment on bone in eugonadal men (or in women) has not been observed, in contrast to known anabolic effects on muscle mass (22). For these reasons and because of concerns about safety, androgen replacement even in hypogonadal men remains a controversial issue (23). Given the modest therapeutic benefit observed with androgen therapy, speculation has arisen that a portion of the positive effect of androgens on bone may be indirectly mediated through increased muscle mass. An increase in lean mass would have beneficial effects on BMD though biomechanical linkage and skeletal adaptation. Consistent with this suggestion, Murphy et al (24) have shown that administration of the synthetic anabolic androgen oxandrolone to severely burned children increases lean body mass three to six months before an increase in bone mineral content is observed.

Analysis of the effects of androgen on the skeleton also should take into consideration the complex nature of bone tissue. Reports have documented the response to systemic androgen administration in distinct skeletal compartments, i.e., cortical, trabecular or intramembranous bone. In hypogonadal settings, a positive response to androgen therapy is generally observed in the trabecular (cancellous) compartment, the more active surface in bone, and bone mass is increased. However, this increase in bone mass is generally observed associated with or as a consequence of inhibition of resorption. Micro-architectural changes also occur consistent with anti-resorptive effects, with an increase in trabecular number but not thickness. Careful analysis of androgen action in orchidectomized male mice has shown that AR activation preserves the

number of trabeculae but does not maintain thickness, volumetric density or mechanical strength (25). Notably, these studies also demonstrated that the bone sparing effect of AR activation is distinct from the bone-sparing effect of ERα. In addition, androgen appears to play an important role in intramembranous bone formation (26). There are also reports of increased cortical mass as a consequence of increased bone width, with surface periosteal expansion (see 13,27). High dose testosterone therapy over 2 years in (genetic female) female-to-male transsexuals resulted in increased areal BMD at the femoral neck and was associated with a non-significant increase in serum osteoprotegerin (OPG) levels, in a setting where estradiol declined to post-menopausal levels (28). In men with constitutional delay of puberty, impaired periosteal expansion is observed (29). Taken together, these findings indicate that androgens act *in vivo* to maintain trabecular bone mass and expand cortical bone at the periosteal surface. While these findings argue that androgen positively affects the skeleton at the periosteal surface (see 30), what is lacking is clear documentation of an anabolic effect on mature osteoblasts and osteocytes to increase bone formation. Thus, the direct consequences of androgen action on differentiated osteoblasts *in vivo* remain unclear, and mechanisms underlying potential positive outcomes on skeletal health controversial.

Concentrations of estrogen and androgen receptors vary during osteoblast differentiation, with AR levels highest in mature osteoblasts and osteocytes (16). Since osteocytes are the most abundant cell type in bone (31), these cells are likely an important target cell for androgen action, and may represent a central mediator for skeletal responses to testosterone therapy *in vivo*. The goal of this study was to use skeletally targeted overexpression of AR in differentiated osteoblasts as a means of elucidating the specific role(s) for AR transactivation in the mature bone compartment. Here

we describe the consequences of enhanced androgen signaling that is restricted to mature osteoblasts and osteocytes, employing the 2.3 Kb type I collagen promoter to control AR overexpression in AR2.3-transgenic mice.

MATERIALS AND METHODS

Cloning of expression plasmids. The pBR327-based plasmid col2.3-ßgal-ClaPa contains the basic rat collagen Iα1 promoter sequence – 2293 to +115 (provided by Dr. David Rowe, University of Connecticut Health Center), which served as the starting vector. *Bam*HI sites were added to the rat AR cDNA (provided by Dr. Shutsung Liao, University of Chicago) with PCR primers. The PCR product was T/A cloned in pCR 2.1-TOPO vector (Invitrogen Corp., Carlsbad, CA). Finally the *Bam*HI-rAR fragment was cloned into the *Bam*HI site in the col2.3-ßgal-ClaPa (after removal of the ßgal cDNA sequences), to give the expression construct termed col2.3-AR (hereafter referred to as the AR2.3-transgene). The correct sequence and orientation of the AR insert was verified by direct DNA sequencing.

Generation of AR2.3 transgenic mice. AR2.3-transgenic mice were produced using standard technology by the Oregon Health & Science University (OHSU) Transgenic Mouse Facility, following methodology previously described (30). Embryos were obtained from matings of C57BL/6 males x DBA/2J females (B6D2F1). Founder mice were identified by PCR genotyping and mated with B6D2F1 (Jackson Labs, Bar Harbor, ME) to produce F2 litters. Transgenic mice were healthy and transmitted the transgene at the expected frequency. The generation and use of transgenic mice were performed according to institutional, local, state, federal and NIH guidelines for the use of animals in research under an Institutional Animal Use and Care Committee-approved protocol.

Animals. AR2.3-transgenic mice were bred to B6D2F1 mice (Jackson Labs, Bar Harbor, ME); both genders were employed. The mice had free access to tap water and were fed a diet

containing 1.14% calcium, 0.8% phosphorous, 2200 IU/kg vitamin D3, 6.2% fat and 25% protein (Purina PMI Nutrition International, St. Louis, MO). All animals were weighed weekly, and body length (nose to rump) was determined at monthly intervals over six months (n = 4-5). For analysis, the animals were sacrificed under CO_2 narcosis by decapitation. The right femur was used for measurement of cortical and trabecular volumetric density and geometry by micro-computed tomography (μCT) *ex vivo,* followed by destructive analysis of whole bone biomechanical properties. The left femur was used for dynamic histomorphometric analysis. The length of the femur was measured from the femoral head to the distal condyles. In addition, a variety of tissues/organs were collected for RNA isolation or histological and immunocytochemical analyses. For RNA isolation, tibia was cleaned of muscle tissue and aseptically dissected. After removal of the epiphyseal area, marrow was briefly flushed with sterile phosphate buffered saline and the bone frozen in liquid nitrogen and stored at –80°C until RNA isolation as described below.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT–PCR) analysis. Total RNA was extracted and the concentration measured at 260 nm using a spectrophotometer, with purity assessed by the $A_{260/280}$ ratio. RNA integrity was confirmed on a 1% agarose gel after SYBR Gold staining (Invitrogen Corp), and qRT-PCR performed as previously described (32). Intron-spanning primers for tibial RNA analysis were purchased pre-designed from Qiagen (Valencia, CA). Transgene-specific RT primers were forward 5'-GCATGAGCCGAAGCTAAC-3' and reverse 5'-GAACGCTCCTCGATAGGTCTTG-3' designed using Oligo Software (Molecular Biology Insights, Inc. Cascade CO), and specifically amplified colAR using sites in the collagen untranslated region and AR near to those used for

genotyping. Fold regulation was determined by normalizing all values to the mean of the relative expression for the control group, or to the value in calvaria.

Serum biochemistry and hormone analysis. Serum specimens from 2-month-old female and male mice of both genotypes were collected and stored at –20°C until analysis was performed (n = 6-17). Blood samples were obtained under anesthesia by cardiac puncture. Serum analysis was as previously described (30), with 17ß-estradiol measured by radioimmunoassay (RIA) using Immuchem Double Antibody 17ß-Estradiol RIA (ICN Biomedicals Inc., Costa Mesa, CA); testosterone measured by enzyme linked immunoassay from Diagnostic Automation Inc. (Calabasas, CA); osteocalcin quantitated by ELISA (Biomedical Technologies Inc., Stoughton, MA); and OPG determined by OPG immunoassay kit (R&D systems, Minneapolis, MN).

Histochemical analysis of calvaria. Histochemical analysis by H&E staining was performed on representative calvaria as previously described (30), from offspring of two independent founder lines (219 and 223). Calvaria were fixed, decalcificied in Immunocal (Decal Corp., Tallman, NY), and sections were processed by dehydration, paraffin infiltration and embedding. Tissue sections (5-6 µm) were cut, processed and subjected to immunohistochemical staining after incubation in a primary antibody directed against AR N-terminus (ab3510, 4 µg/ml; Abcam Inc, Cambridge, MA) at 4 °C overnight. For AR detection, sections were incubated for 1 h in a biotinylated goat antirabbit secondary antibody (1: 200; Vector Laboratories, Burlingame, CA, USA). Following rinses, sections were incubated for 60 min in avidin-biotin complex (1: 1000; Vectastain Elite; Vector Laboratories). After 30 min of rinsing, sections were incubated for 10 min with a

diaminobenzidine (DAB) solution (0.05% DAB) activated by 0.001% hydrogen peroxide. Slides were counterstained with hematoxylin.

Dynamic histomorphometry. Bone formation and resorption during the last week of growth was assessed by dynamic histomorphometric measures after flurochrome labeling (n = 8-20 males; 10-15 females). Prior to sacrifice, 2-month-old mice received two fluorochrome labels by intraperitoneal (ip) injection [oxytetracycline hydrochloride (Sigma, St. Louis, MO) at 30 mg/kg and calcein green (Sigma) at 10 mg/kg], given 10 days and 3 days before death, respectively. Left femora were dissected and processed non-decalcified for plastic embedding as previously described (33). Cross sections (100 μm) through mid-diaphysis were prepared using a diamond-wafering saw (Buhler, Lake Bluff, IL USA), then polished to a thickness of 30 microns. Sections were left unstained and dynamic histomorphometry was carried out using a light/epifluorescent microscope and a semiautomatic image analysis system (OsteoMetrics, Inc., Decatur, GA USA). Standard measures of bone formation and resorption including mineral apposition rate (MAR, μm/day), mineralizing perimeter (Min.Pm.), bone formation rate (BRF) and eroded surface were determined for both periosteal and endosteal surfaces. In addition, the cross-sectional area, cortical area, cortical thickness, and endosteal and periosteal perimeters were measured on cortical cross-sections. The terminology and units used were those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (34).

Bone morphology and microstructure. The morphological consequences of increased AR expression in osteoblastic/osteocytic cells in AR2.3-transgenic animals were evaluated in 2-

month-old male and female mice by dual energy x-ray absorptiometry (DXA) and quantitative μCT. BMD, bone mineral content (BMC) and body composition was measured by whole body DXA using a mouse densitometer (PIXImus2, Lunar, Madison WI, USA). Right femurs from each genotype (n = 10-21 males; 13-19 females) were examined for diaphyseal cross-sectional morphology and tissue mineral density (TMD) using an eXplore Locus SP Pre-Clinical Specimen MicroComputed Tomography system (GE Medical Systems, London, Ontario) as described previously (35). Area measures were body weight adjusted to reduce variability. Three-dimensional images of the entire femur were obtained at an 8.7 μm voxel size and individually thresholded using a standard segmentation algorithm (36). A 3mm region of the reconstructed mid-diaphysis, corresponding to the typical failure region for 4-point bending (see below), was examined. Determination of cross-sectional morphology was performed using custom analysis program (MathWorks, v. 6.5; The MathWorks, Inc., Natick MA, USA) (35). Trabecular morphometry of the distal metaphysic, including bone volume fraction (BV/TV) and trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp), was assessed using Microview Advanced Bone Analysis (GE Medical Systems, v. 1.23).

Images from the μCT analysis were also used to quantify TMD as described previously (35). TMD is the average mineral value of the bone voxels alone, expressed in hydroxyapatite (HA) density equivalents. TMD was calculated by converting the grayscale output of bone voxels in Hounsfield units (HU) to mineral values (mg/cc of HA) through the use of a calibration phantom hydroxyapatite (SB3: Gamex RMI, Middleton, WI, USA) [TMD = average bone voxel HU / average HA phantom HU * 1130 mg/cc (HA physical density)] (35). The same calibration

phantom was used for all scans to normalize mineral density measurements and to account for possible variability among scan sessions.

Mechanical testing. Following DXA and μCT analysis, the right femurs were subjected to destructive testing to establish whole bone mechanical properties. Femurs were loaded to failure in 4-point bending. All whole bone-bending tests were conducted by loading the femurs in the posterior to anterior direction. The load-deflection curves were analyzed for stiffness (the slope of the initial portion of the curve), maximum load, post-yield deflection (PYD), and work-to-failure as described previously (33).

Statistical analysis. All data were analyzed using Prism software v4 (GraphPad Software, Inc., San Diego, CA). Significance of difference between wild-type and AR2.3-transgenic mice was assessed by an unpaired two-tailed t-test using Welch's correction. Body lengths and weights were analyzed by repeated measures two-way ANOVA for the effects of gender and genotype. All data are shown as mean ± standard error of the mean (SEM).

RESULTS

Generation of transgenic mice with enhanced androgen signaling in mature osteoblasts and osteocytes.

Confusion exists regarding the *in vivo* action of androgens in bone due to metabolism to estrogen, and because androgen influences many tissues in the body and many months of treatment are required to observe improvement in BMD. The AR2.3-transgenic animal model was created to determine the specific physiologic relevance of androgen action in the mature osteoblast/osteocyte population in bone, through tissue-specific overexpression of AR. This line is distinct from our previously generated transgenic model with AR overexpression in stomal precursors, periosteal fibroblasts and throughout the osteoblast lineage, the AR3.6-transgenic line (30). A transgene cassette (AR2.3) was cloned as described in Materials and Methods and AR2.3-transgenic mice were created following standard procedures. Positive founders were identified by PCR genotyping and were bred to wild-type B6D2F1 mice; two AR2.3-transgenic lines (lines 219 and 223) derived from independent founders have been retained. Southern analysis confirmed a single insertion site for the AR2.3-transgene (data not shown). Table 1 lists the qRT-PCR analysis of expression of the AR2.3-transgene in various tissues, showing the expected bone targeting with highest levels in calvaria but ~100-3000 fold lower levels in muscle, skin, heart, intestine, kidney, liver, lung and spleen.

Phenotype in AR2.3-transgenic mice with bone-targeted AR overexpression.

To begin to characterize the phenotype of AR2.3-transgenic mice, we first determined body weight gain and nose-rump length over a 6-month period. At birth, animals were indistinguishable and as the mice aged, AR2.3-transgenic males and females gained length and weight similar to wild-type

controls (Fig. 1A, B). Body composition and bone density were evaluated by DXA at 2 months in male and female AR2.3-transgenic mice and wild-type littermate controls (Fig. 1C). While systemic androgen treatment is known to affect body compositional changes, no difference was noted in either lean mass or fat mass in either males or females (Fig. 1C, D), consistent with skeletal targeting of the AR transgene. In addition, areal BMD was not affected (Fig. 1E), indicating a lack of effect on periosteal surfaces. However, BMC showed a trend for reduction in male transgenic mice (Fig. 1F).

AR2.3-transgenic mice were next evaluated for serum biochemistry and hormone levels at 2 months of age. As expected because of bone-targeting of AR overexpression, serum testosterone and estradiol levels were not significantly different between littermate controls and transgenic animals in either sex (Fig. 2A, B). There were also no significant differences in serum calcium levels between transgenic mice and littermate controls (Fig. 2C). Interestingly, there was a significant ~50% decrease in serum osteocalcin levels in male AR2.3-transgenic animals but not in females ($P < 0.01$, Fig. 2D). Serum OPG, an important inhibitor of osteoclastogenesis (37), was also analyzed. A modest but non-significant increase in serum OPG was observed in AR2.3-transgenic males, again with little effect in transgenic females compared to littermate controls (Fig. 2E).

To further evaluate the phenotype of AR2.3-transgenic mice, we characterized AR protein expression *in vivo* by immunocytochemical analysis in calvarial sections from both sexes. In this analysis, immunostaining represents combined AR levels with detection of both endogenous AR and the product of the AR2.3-transgene. In transgenic mice, the majority of osteoblasts and osteocytes demonstrated AR immunoreactivity (Fig. 2F, left panels), consistent with increased AR2.3-transgene expression. Both male and female transgenic animals revealed higher level of AR

expression, with no notable difference between the sexes or between independent families (data not shown). Morphological changes were characterized by H&E staining (Fig. 2F right panels). Again, there was no difference between wild-type and AR2.3-transgenic mice of either gender, nor between the independent AR2.3-transgenic families 219 and 223 (data not shown). In contrast to these findings, it is noteworthy that the calvaria from male AR3.6-transgenic mice, with AR overexpression in periosteal fibroblasts, demonstrate substantial calvarial thickening (Fig. 2F right panels).

Enhanced androgen signaling results in altered bone morphology and reduced cortical area in male transgenic mice.

Overall bone morphology and femoral structure were quantified from high resolution μCT images. Measures from the μCT analysis for morphological assessment are described in Fig. 3A. No effect of AR2.3-transgene expression on total cross-sectional area or surface periosteal perimeter was observed in either males or females (Fig. 3B, E). However, marrow cavity area was significantly increased in transgenic males (*i.e.*, reduced infilling; Fig. 3C). Given no compensatory changes in the periosteal layer, this inhibition results in a modest but significant reduction in cortical bone area (Fig. 3D) in male AR2.3-transgenic mice. Thus, enhanced AR signaling in mature osteoblasts has significant inhibitory effects on overall femoral cortical bone area. This morphological difference at the diaphysis was not observed in female transgenic mice. We also evaluated polar moment of inertia and tissue mineral density (TMD) at the mid-diaphysis. There was no significant effect on polar moment of inertia in either male or female AR2.3-transgenic mice (Fig. 3F), but males show a significant reduction in TMD ($P < 0.001$; Fig. 3G), consistent with the reduction in BMC (shown in Fig. 1F).

Reduced endosteal bone formation with bone-targeted AR overexpression.

Because of the changes in cortical bone area observed in the μCT analysis in males, dynamic histomorphometric analysis using fluorescent imaging was carried out at the femoral diaphysis. Fluorochromes were administered (oxytetracycline followed by calcein) to label new mineral deposition. Figure 3H shows patterns of bone formation in images of fluorochrome labeling from femoral cross sections. The AR2.3-transgenic males (upper panel) demonstrate a dramatic lack of labeling at the endosteal surface compared with wild-type controls (lower panel). Consistent with these fluorescent images, quantitative dynamic histomorphometric analysis revealed inhibitory responses at the endosteal surface. Dramatic inhibition (~70%) of both BFR ($P < 0.001$; Fig. 3I) and MAR ($P < 0.001$; Fig. 3J), a measure of osteoblast vigor, were noted at the endosteal surface. Eroded surface was reduced at the periosteal surface ($P < 0.001$; Fig. 3K), and labeled perimeter (L.pm) showed inhibition at the endosteal surface ($P < 0.01$; Fig. 3L) but stimulation at the periosteal surface ($P < 0.01$). The modest increase in periosteal activity does not parallel changes in cortical bone morphology that were observed by μCT analysis in Figure 3E, likely since the labeling is representative of mineralization patterns only for the period of time that the labels are present.

Ability to resist fracture is impaired in male AR2.3-transgenic mice.

To analyze whole bone biomechanical and failure properties, femurs from 2-month-old wild-type and AR2.3-transgenic animals were loaded to failure in 4-point bending at 0.05 mm/s. Although overall geometry of the femur demonstrated no obvious differences between wild-type and transgenic mice (Fig. 4A), failure properties were significantly impaired. Diaphyseal strength as

maximum load ($P < 0.05$, Fig. 4B) and stiffness ($P < 0.05$, Fig. 4C) were decreased by about 10% in male AR2.3-transgenic mice (with no change in females), consistent with the decreased cortical bone area in these mice (see Fig. 3C). Male transgenic bones, however, were dramatically impaired in their ability to resist fracture. They were more brittle, with an approximately 40% decrease in post-yield deflection ($P < 0.05$, Fig. 4D), and work-to-failure ($P < 0.05$, Fig. 4E) was reduced by nearly 30% compared to wild-type control bones. Male AR2.3-transgenic mice in this cohort showed no difference in femoral length or weight (Fig. 4F, G)

AR overexpression leads to increased trabecular bone volume in male transgenic mice.

To determine the consequences of AR overexpression on the trabecular bone compartment, we used μCT analysis. Visualization of trabecular bone in the metaphysis after manual subtraction of the cortical shell shows that trabecular bone volume is increased in male AR2.3-transgenic mice (Fig. 5A). To better characterize trabecular micro-anatomy and architecture, static histomorphometric analysis was performed from images of the metaphyseal trabecular region (Fig. 5B-E). Male AR2.3-transgenic mice showed a ~50% increase in trabecular bone volume as a percent of tissue volume (BV/TV; $P < 0.05$; 5B), consistent with the μCT image. The increase in trabecular bone volume was associated with an ~25% increase in trabecular number (Tb.N; $P < 0.01$; 5C), no effect on trabecular thickness (Tb.Th; 5D), and thus a ~30% decrease in spacing (Tb.Sp; $P < 0.01$; 5E).

Enhanced androgen signaling in mature osteoblasts leads to reduced expression of molecular markers of bone formation and osteoclast activation in cortical bone.

Lastly, we analyzed gene expression in long bone from wild-type and AR2.3-transgenic mice of both

genders. Differences in gene expression in RNA isolated from tibial mid-diaphysis were determined by qRT-PCR analysis and included genes important in both osteoblast and osteoclast activity/development (Fig. 6). Osteoblastic genes evaluated were cyclin D1, osterix, type I collagen (col), and osteocalcin (OC). Levels of osteoblastic genes are listed in an order reflecting their temporal expression patterns during osteoblast differentiation, e.g., osteocalcin is expressed late in osteoblast differentiation. Osteoclastic genes analyzed were OPG, receptor activator of NF-κB ligand (RANKL), tartrate-resistant acid phosphatase (TRAP), cathepsin K (CatK) and calcitonin receptor (CTR). Interestingly, the level of inhibition of expression of osteoblastic genes in mid-diaphyseal tissue from male transgenic mice increased with the differentiation stage, from proliferating osteoblasts to osteocytes, and suggest alteration of the organic matrix. This pattern mirrors the pattern of AR overexpression under control of the col2.3 promoter. Inhibition of gene expression was also observed in osteoclast genes in male AR-transgenic mice, with the exception of the increase in OPG expression (secreted from osteoblasts). Both the modest increase in OPG as well as the reduction in osteocalcin expression in transgenic males are comparable to the results observed for serum levels of these proteins (see Fig. 2E and 2D, respectively). Consistent with the lack of a bone phenotype in females, there was little difference in expression in female transgenics compared to control mice for any of the osteoblastic or osteoclastic genes analyzed.

DISCUSSION

The specific role of the AR in maintenance of skeletal homoeostasis remains controversial. To determine the specific physiologic relevance of androgen action in the mature osteoblast/osteocyte population in bone, mice with targeted AR overexpression in mature osteoblasts were developed. Characterization of the consequences of bone-targeted overexpression revealed a skeletal phenotype in male transgenic mice versus littermate controls, with little difference between the females. Collectively, the phenotype observed in male transgenic mice is likely dependent on the higher levels of androgen (~10-fold) circulating in males *vs*. females. In this study we have found that AR overexpression in the mature osteoblast population *in vivo* results in a low turnover state with increased trabecular bone volume, but a significant reduction in cortical bone area due to inhibition of bone formation at the endosteal surface and a lack of marrow infilling. Combined, our results indicate that enhanced androgen signaling in mature osteoblasts/osteocytes inhibits cortical bone formation, and results in changes that are detrimental to matrix quality, biomechanical competence and whole bone strength.

Among the most striking biomechanical characteristics of the bone from AR2.3-transgenic mice was its markedly impaired fracture resistance. Long bones were significantly more brittle and consequently showed large decreased in work-to-failure. The inhibition in bone quality appears to be principally determined by changes in the organic matrix of bone, through the ~50% reduction in MAR at the endosteal surface (which reflects osteoblast vigor or work), leading to a detrimental change in the composition of the material properties of the organic matrix and thus a worsening in PYD and work-to-failure. The increased brittleness (decreased post-yield deflection) of cortical bone is not likely due to over-mineralization, since both TMD and BMC were reduced in

transgenic males, not increased. Brittleness, and its opposite, ductility, are functional attributes that in bone derive principally from matrix composition and collagen organization rather than bone geometry and mass, which are the major determinants of bone stiffness and strength. Thus, the brittleness observed in these AR transgenic mouse bones points to a defect in bone matrix quality. This in turn suggests a defect in osteoblast production of a functionally appropriate bone matrix in the presence of enhanced androgen signaling in mature osteoblasts/osteocytes. Indeed, our molecular analysis of expression differences from the AR transgenic mice show dramatically reduced collagen and osteocalcin production, consistent with the impaired matrix quality in these mice.

An important advantage of the AR2.3-transgenic mouse model is the enhancement of androgen signaling as a consequence of increased AR abundance in a likely target for androgen *in vivo*, i.e., mature osteoblasts and osteocytes. This model, characterized by the absence of differences in circulating testosterone or 17ß-estradiol and studied without systemic androgen administration, thus takes advantage of increased sensitivity to androgen in distinct skeletal sites for the analysis of compartment-specific effects of androgen. At the same time, AR overexpression, rather than systemic administration, excludes action at other androgen target tissues *in vivo* including muscle and fat. AR overexpression targeted by the col2.3 promoter was chosen for several reasons: the skeletal expression patterns for this promoter are both well-characterized and bone-selective (see 38,39); the col2.3 promoter is not active in the periosteum but is strongly expressed in osteocytes and mineralizing nodules (40); the col2.3 promoter is also not active in osteoclasts; and androgens do not inhibit expression from the 2.3 kb promoter fragment (data not shown). Thus, the col2.3 promoter fragment directs expression of the fused transgene in bone, with strong expression still observed at

the age of 3 months and even in animals as old as 6 months (40). Reports describing characterization of expression indicated strong expression in cells at osteogenic fronts of parietal bones, but the suture area was negative. In long bones, strong transgene expression was observed in most osteoblasts on endosteal surfaces, and in a large proportion of osteocytes in femurs throughout cortical bone, with no expression seen in periosteal fibroblasts (41). In the trabecular area of metaphyseal bone, strong expression was observed at all developmental stages (40).

It is instructive to compare the skeletal phenotypes that develop in the two distinct lines that we have generated, the AR2.3-transgenic mice described in these studies and the previously characterized AR3.6-transgenic model (30). In broad terms, the skeletal phenotype characterized in AR2.3-transgenic mice mirrors that described previously for AR3.6-transgenic males, indicating the specificity and reproducibility of the phenotypic consequences of bone-targeted androgen signaling. In common between the two models, we have shown increased trabecular bone volume, reduced formation at endosteal surfaces, reduced bone turnover and compromised biomechanical strength in male transgenic mice. With the exception of enhanced periosteal activity in AR3.6-transgenic males as noted below, neither model exhibits anabolic responses in the cortical bone compartment and instead both show inhibition of bone formation at the endosteal surface and compromised biomechanical properties. By comparing and contrasting the two AR-transgenic models, we propose that the commonalities in the bone phenotype between AR2.3-transgenic and AR3.6-transgenic mice arise from AR overexpression in mature osteoblasts and osteocytes, since both promoters are active in these cells. Thus, the increased trabecular bone volume, reduced bone turnover, reduced formation and decreased osteoblast vigor at endosteal surfaces, and compromised biomechanical strength with increased bone fragility observed in both

models, are likely to be mediated at least in part by enhanced androgen signaling in mature osteoblasts/osteocytes.

The most striking contrast between the two AR-transgenic models we have developed is observed at periosteal surfaces in AR3.6-transgenic males, which show increased cortical bone formation in the periosteum and dramatic intramembranous calvarial thickening. This finding was expected, given col3.6 transgene targeting to the periosteum and, conversely, the lack of expression at the same compartment with col2.3 transgene expression. The specificity of the periosteal anabolic effect in AR3.6-transgenic males is consistent with previous reports documenting the importance of androgen signaling in periosteal expansion (42). Periosteal bone formation defines the cross sectional area of bone or bone width, whereas endosteal formation or resorption determines cortical thickness. During development, girls and boys build mechanically functional structures (i.e., the size, shape and quality of the bone appears to be well matched for the size of the individual). However, they get there by different means (43). Before puberty, boys and girls grow in much the same way but during/after puberty, the increase in estrogen in girls leads to reduced periosteal expansion and then a reversal on the endosteum from expansion to infilling. In boys, testosterone levels increase and in contrast to girls, lead to further expansion of the periosteum but also continued expansion of the endosteal cavity. Consequently, the outer diameter of girls' bones tends to be smaller than that of boys' bones and greater cross sectional area is observed in males (44), yet cortical thickness is similar between males and females (45, but see 46) because of adaptive infilling in females. Thus, we propose that androgen inhibition of medullary bone formation at the endosteal surface in males may subserve an important physiological adaptive function, being key for appropriate spatial distribution and maintenance of the total amount/weight

of bone in the cortical envelope. A reasonable hypothesis is that androgens strongly promote the addition of cortical width through periosteal growth, but balance that growth with inhibition in the marrow cavity so that the skeleton does not become too heavy (see 47). Based on our characterization of AR-transgenic mouse models and other published reports, we propose a model for the consequences of androgen signaling where the effects of AR activation are distinct in different skeletal compartments (Fig. 7). Thus, in trabecular bone, androgens reduce bone turnover but increase trabecular volume through an increase in trabecular number. In cortical bone, androgens inhibit osteogenesis at endosteal surfaces but increase bone formation at periosteal sites (30), to maintain cortical thickness yet displace bone further away from the neutral axis in males. Androgens also positively influence bone at intramembranous sites (26,30). In addition, androgen administration increases muscle mass, partially mediated by effects on mesenchymal stem cell lineage commitment (48), likely to indirectly influence bone density through biomechanical linkage. Additional studies will be needed to more fully test these hypotheses.

Male AR-transgenic mice also demonstrate a phenotype consistent with reduced osteoclast resorptive activity. TRAP and RANKL expression is reduced, with an increase in OPG, an important negative regulator of osteoclast differentiation, survival and activation (49). In addition, the increase in trabecular bone volume with a decrease in trabecular separation observed is a hallmark of antiresorptive activity. Potential modulation of osteoclast action by DHT is incompletely characterized, although there are reports of AR expression in the osteoclast (17). The effect of androgen is undoubtedly complex, given data that androgens may inhibit levels of OPG (50,51), although previously we (30) and others (52) have shown that androgen can stimulate OPG levels. Although androgen may be a less significant determinant of bone resorption *in vivo* than

estrogen (53), this remains controversial (54). The bone phenotype that develops in a global AR null male mouse model, a high-turnover osteopenia with reduced trabecular bone volume and a stimulatory effect on osteoclast activity (55-57), also supports the importance of androgen signaling through the AR to influence resorption, and is generally opposite to the phenotype we observe with targeted AR overexpression. Interestingly, the global AR null model also develops late onset obesity (58). Finally, recent publications document that androgen reduces bone resorption of isolated osteoclasts (59), inhibits osteoclast formation stimulated by PTH (60), and may play a direct role regulating aspects of osteoclast activity in conditional AR null mice (61). Our results suggest that at least some component of inhibition of osteoclastic resorptive activity as a consequence of androgen administration is mediated indirectly through effects on mature osteoblasts and osteocytes.

Some of the negative consequences of AR overexpression in mature osteoblasts we have observed *in vivo* may reflect previously documented *in vitro* reports. For example, there are reports, some in clonal osteoblastic cell lines, of effects of gonadal androgen treatment on differentiation, matrix production and mineral accumulation mediated by AR signaling (62-64). These findings are variable however, with other reports of no effect or even inhibition of osteoblast markers (65-67), consistent with our gene expression analysis in AR-transgenic mice. In addition, the effect of androgens on osteoblast proliferation is controversial. We have previously demonstrated that either stimulation or inhibition of osteoblast viability by androgen can be observed, and these effects are dependent on the length of treatment. Transient administration of nonaromatizable DHT can enhance transcription factor activation and osteoblast proliferation, while chronic treatment inhibits both mitogenic signaling and MAP kinase activity (68). Chronic DHT treatment *in vitro* can also enhance osteoblast

apoptosis (69). Thus, these *in vitro* reports are consistent with the detrimental changes in matrix quality and osteoblast vigor we observe in the AR-transgenic model *in vivo*.

In summary, complex skeletal analysis using morphological characterization by μCT, dynamic and static histomorphometric analysis, DXA, biomechanical testing and gene expression studies all indicate that androgen signaling in mature osteoblasts inhibits osteogenesis at endosteal surfaces and produces a low turnover state; these changes are detrimental to overall matrix quality, biomechanical competence, bone fragility and whole bone strength. It is possible that the observed inhibition of osteogenesis and lack of anabolic response, as a consequence of enhanced androgen signaling in mature bone cells, underscores an important physiological function for androgen in the skeleton: to maintain an appropriate spatial distribution of bone in the cortical envelope. The strong inhibition of bone formation at the endosteal surface and increase in bone fragility may also underlie the limited therapeutic benefits observed with androgen therapy, as noted above. Because of the detrimental consequences of direct androgen signaling in bone, these results raise concerns regarding anabolic steroid abuse or high dose androgen therapy during growth and in healthy eugonadal adults.

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TABLE 1

Analysis of transgene expression in a variety of tissues from AR2.3-transgenic mice.

Tissues listed were harvested from male AR2.3-transgenic mice and total RNA was isolated (n = 5). Expression of the transgene was evaluated by real-time qRT-PCR analysis after normalization to the total RNA concentration using RiboGreen (32). Data are expressed relative to the expression level in calvaria as mean ± SEM. n.a., not applicable; n.d., not detectable.

Tissue	AR2.3-tg level	Fold difference
Calvaria	1.0000 ± 0.2373	n.a.
Thymus	0.0066 ± 0.0011	-152
Lung	0.0054 ± 0.0005	-185
Heart	0.0047 ± 0.0019	-213
Kidney	0.0044 ± 0.0006	-227
Fat	0.0027 ± 0.0007	-370
Spleen	0.0025 ± 0.0004	-400
Muscle	0.0006 ± 0.0000	-1667
Skin	0.0006 ± 0.0002	-1667
Ear	0.0004 ± 0.0001	-2500
Liver	0.0004 ± 0.0002	-2500
Tendon	0.0003 ± 0.0000	-3333
Intestine	0.0000 ± 0.0000	n.d.

FIGURE LEGENDS

Figure 1. Weight changes and body composition analysis in AR2.3-transgenic mice.

Body weight and nose-rump-length determinations were carried out weekly or monthly, respectively, over six months in both genders in both wild-type (wt) and AR2.3-transgenic (AR2.3-tg) mice. **A**. Weight gain in growing male (left) and female (right) mice. Analysis for the effects of time and genotype by repeated measures two-way ANOVA in males revealed an extremely significant effect of time ($F = 218.36$; $P < 0.0001$) but not genotype, and with no interaction; females were similar ($F = 114.80$; $P < 0.0001$). **B**. Nose-rump length in male (left) and female (right) mice. Analysis by repeated measures in males revealed an extremely significant effect of time ($F = 228.54$; $P < 0.0001$) and an effect of genotype ($F = 15.87$; $P < 0.01$) with no interaction; females only showed an effect of time ($F = 149.48$; $P < 0.0001$). Data is shown as mean ± SEM, n = 4-5. DXA was performed on 6-month-old AR2.3-transgenic and littermate control mice to assess bone mineral, lean mass and fat mass. **C**. Lean mass adjusted for total tissue mass. **D**. Fat mass adjusted for total tissue mass. **E**. Areal BMD (minus head). **F**. BMC. A trend for decreased BMC was noted in male AR2.3-tg mice ($p=0.0571$). Values are expressed as mean ± SEM, n = 4-10.

Figure 2. **Phenotypic characterization of serum markers and calvarial thickness in AR2.3-transgenic animals**.

Comparisons were performed between wild-type littermate control (wt) and AR2.3-transgenic (AR2.3-tg) animals. Serum from 2-month-old mice was analyzed to determine levels of hormones and markers of calcium metabolism. Assays were performed in duplicate by RIA for 17ß-estradiol or EIA for testosterone, OPG and intact mouse osteocalcin, and for calcium by the

colorimetric cresolphthalein-binding method. **A**. Testosterone. **B**. 17ß-estradiol. **C**. Calcium. There were no statistical differences between the genotypes for 17ß-estradiol, testosterone or calcium levels. **D**. Osteocalcin levels were significantly reduced in male AR2.3-tg mice. **E.** OPG circulating levels were elevated in males but not in female AR2.3-tg mice. Values are expressed as mean ± SEM, n=6-17. **, $P < 0.01$ (*vs*. gender-appropriate wild-type control). **F**. Histological and immunohistochemical analysis of calvaria isolated from 2-month-old mice. Sections were subjected to either H&E staining or immunohistochemical staining after demineralization and paraffin embedding. Representative sections are shown. **Left**. AR abundance was visualized with rabbit polyclonal antisera for male and female mice from wt and AR2.3-tg mice. AR is brown and the nucleus is purple after after DAB incubation and counterstaining with hematoxylin. The majority of osteoblasts and osteocytes demonstrated AR immunoreactivity. **Right.** Calvaria from male wild-type, AR2.3-tg or AR3.6-tg mice were evaluated for increased thickness and periosteal bone formation. Scale bar = 50 µm.

Figure 3. Cortical morphology, structural analysis and bone formation rates in AR2.3-transgenic mice.

Femurs were isolated from 2-month-old male and female wild-type (wt) or AR2.3-transgenic mice (tg) and subjected to high resolution µCT imaging at mid-diaphysis. **A.** Parameters for morphological characterization by µCT. **B**. Total cross-sectional area. **C**. Marrow cavity area. **D**. Cortical bone area. **E**. Periosteal perimeter. **F**. Polar moment of inertia. **G**. Tissue mineral density. Values are shown as mean ± SEM, n = 10-21 males; 13-19 females. Differences between genotypes were determined by Student's unpaired *t*-test with Welch's correction. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs*. gender-appropriate wt controls). For dynamic histomorphometric

analysis, male femurs were sectioned at the mid-diaphysis; rates were determined at both the endosteal and periosteal surfaces. **H**. Fluorescent images of femur after double-label administration. Representative photomicrographs are shown with higher power insets demonstrating labeling on the endosteal surface. Bands were photographed at comparable anatomic positions for each bone. **I**. Bone formation rate (BFR). **J**. Mineral apposition rate (MAR). **K**. Percent eroded surface. **L**. Percent labeled perimeter (L.pm). Values are shown as mean ± SEM; n = 8-20 males; 10-15 females. Differences between genotypes were determined by Student's unpaired *t*-test with Welch's correction. **, $P < 0.01$; ***, $P < 0.001$ (*vs*. wt controls). Scale bar = 200 µm in figure; scale bar = 100 µm in insets as indicated.

Figure 4. Whole bone strength and failure properties determined from biomechanical analyses.

Femurs from 2-month-old male and female wild-type (wt) and AR2.3-transgenic (tg) mice were loaded to failure in 4-point bending analysis. Stiffness, maximum load, and post-yield deflection were calculated from the load-deflection curves. Stiffness and maximum load are adjusted for body weight differences. **A**. Whole bone morphology from µCT imaging. **B**. Stiffness Adjusted. **C**. Maximum Load Adjusted. **D**. Post-yield deflection. **E**. Work-to-failure. **F**. Femoral Length. **G**. Body weight in cohort. Whole bone biomechanical properties are shown as mean ± SEM, n = 10-21 males; 13-19 females. Differences between genotypes were determined by Student's unpaired *t*-test with Welch's correction. *, $P < 0.05$ (*vs*. gender-appropriate wt controls).

Figure 5. Trabecular morphology and micro-architecture in AR2.3-transgenic mice.

Computer-aided analysis of μCT images was used to derive measures of trabecular bone micro-architecture in the metaphysis of 2-month-old male and female wild-type (wt) or AR2.3-transgenic (tg) mice. Measurements included trabecular bone volume as a percent of tissue volume (BV/TV); trabecular number, spacing, and thickness (Tb.N, Tb.Sp, Tb.Th). **A**. Reconstructed images were evaluated for trabecular morphology in the distal metaphysis. **B**. BV/TV. **C**. Tb.N. **D**. Tb.Th. **E**. Tb.Sp. Values are expressed as mean ± SEM, n = 10-21 males; 13-19 females. Differences between genotypes were determined by Student's unpaired *t*-test with Welch's correction. *, $P < 0.05$; **, $P < 0.01$ (*vs*. gender-appropriate wt controls).

Figure 6. Molecular markers of osteoblasts and osteoclasts in cortical bone by qRT-PCR analysis.

Analysis of steady-state mRNA expression characteristic of osteoblastic or osteoclastic cells was determined by real-time qRT-PCR analysis using tibial RNA isolated from male and female wild-type (wt) or AR2.3-transgenic mice (tg). Osteoblast genes involved in bone formation and matrix production examined included cyclin D1, osterix, type I α_1 collagen (Col), and osteocalcin (OC). Genes involved osteoclastic activity and bone resorption were osteoprotegerin (OPG), RANK ligand (RANKL), tartrate-resistant acid phosphatase (TRAP), calcitonin receptor (CTR) and cathepsin K (CatK). **A.** Determination of osteoblast gene expression in males and female mice. **B.** Analysis of osteoclastic gene expression in males and females. n = 4-5 males; 2-8 females. Values are expressed as mean ± SEM.

Figure 7. Model for androgen action in the skeleton mediated by AR transactivation.

Androgen activation of AR influences a variety of target organs and skeletal sites, including

marrow stromal cells, and trabecular, cortical and intramembranous bone compartments. Arrows indicate the changes associated with androgen action. In trabecular bone, androgen action preserves or increases trabecular number, has little effect on trabecular thickness, and thus reduces trabecular spacing. In cortical bone, AR activation results in reduced bone formation at the endosteal surface but stimulation of bone formation at the periosteal surface; correspondingly decreased periosteal but increased endosteal resorption results in no change in cortical area. Summary based on results presented here and references cited in the text. In the transgenic model, AR activation in mature bone cells *in vivo* results in a low turnover phenotype, with inhibition of bone formation and inhibition of gene expression in both osteoblasts and osteoclasts. In the absence of compensatory changes at the periosteal surface, these changes are detrimental to overall matrix quality, biomechanics and whole bone strength.

Figure 1

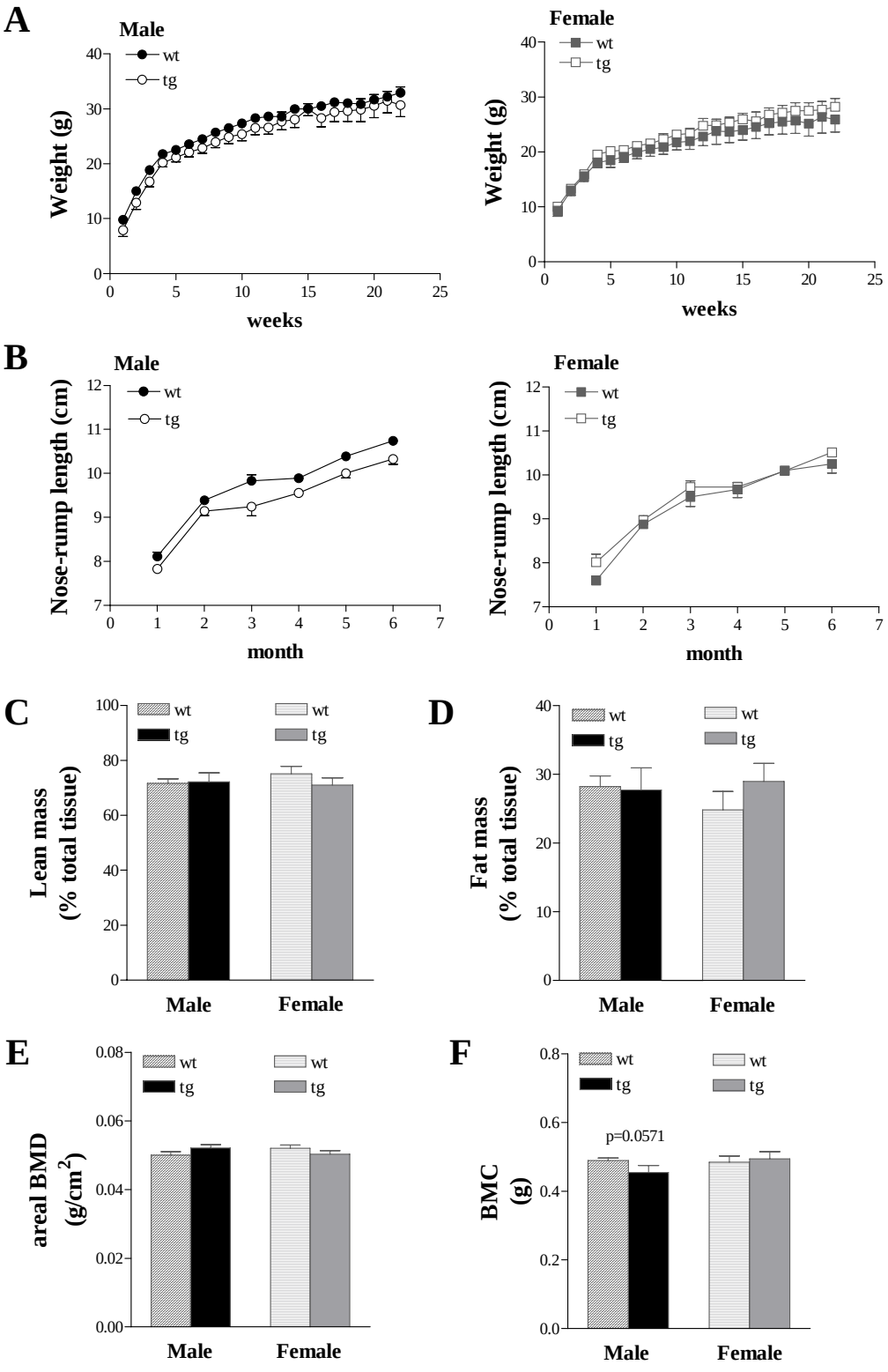

A
Male
wt
tg
Weight (g)
weeks
Female
wt
tg
Weight (g)
weeks
B
Male
wt
tg
Nose-rump length (cm)
month
Female
wt
tg
Nose-rump length (cm)
month
C
wt
tg
wt
tg
Lean mass (% total tissue)
Male
Female
D
wt
tg
wt
tg
Fat mass (% total tissue)
Male
Female
E
wt
tg
wt
tg
areal BMD (g/cm2)
Male
Female
F
wt
tg
wt
tg
p=0.0571
BMC (g)
Male
Female

Figure 2

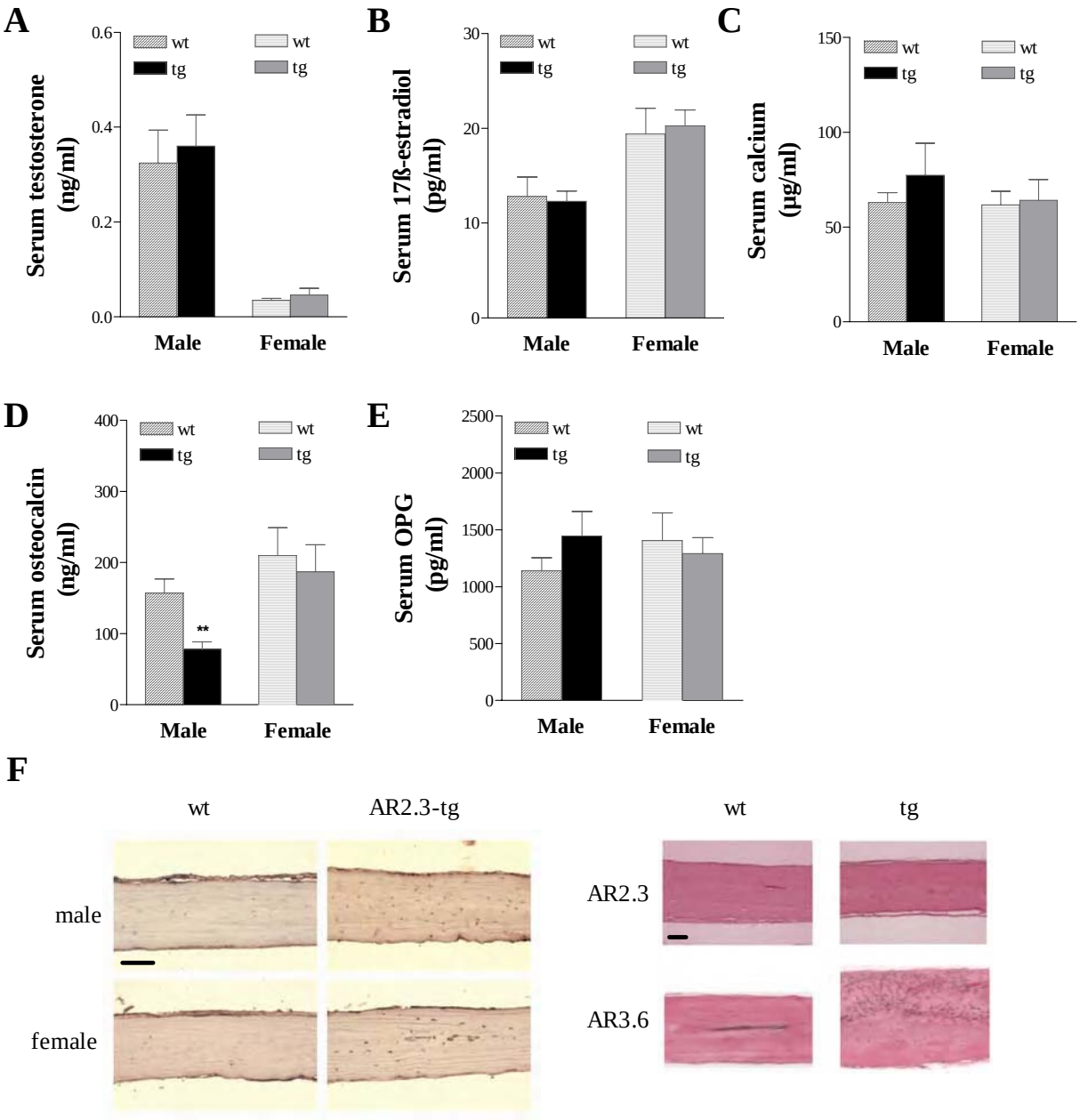

A
Serum testosterone (ng/ml)
0.6
0.4
0.2
0.0
wt
tg
wt
tg
Male
Female
B
Serum 17ß-estradiol (pg/ml)
30
20
10
0
wt
tg
wt
tg
Male
Female
C
Serum calcium (µg/ml)
150
100
50
0
wt
tg
wt
tg
Male
Female
D
Serum osteocalcin (ng/ml)
400
300
200
100
0
**
wt
tg
wt
tg
Male
Female
E
Serum OPG (pg/ml)
2500
2000
1500
1000
500
0
wt
tg
wt
tg
Male
Female
F
wt
AR2.3-tg
male
female
wt
tg
AR2.3
AR3.6

Figure 3

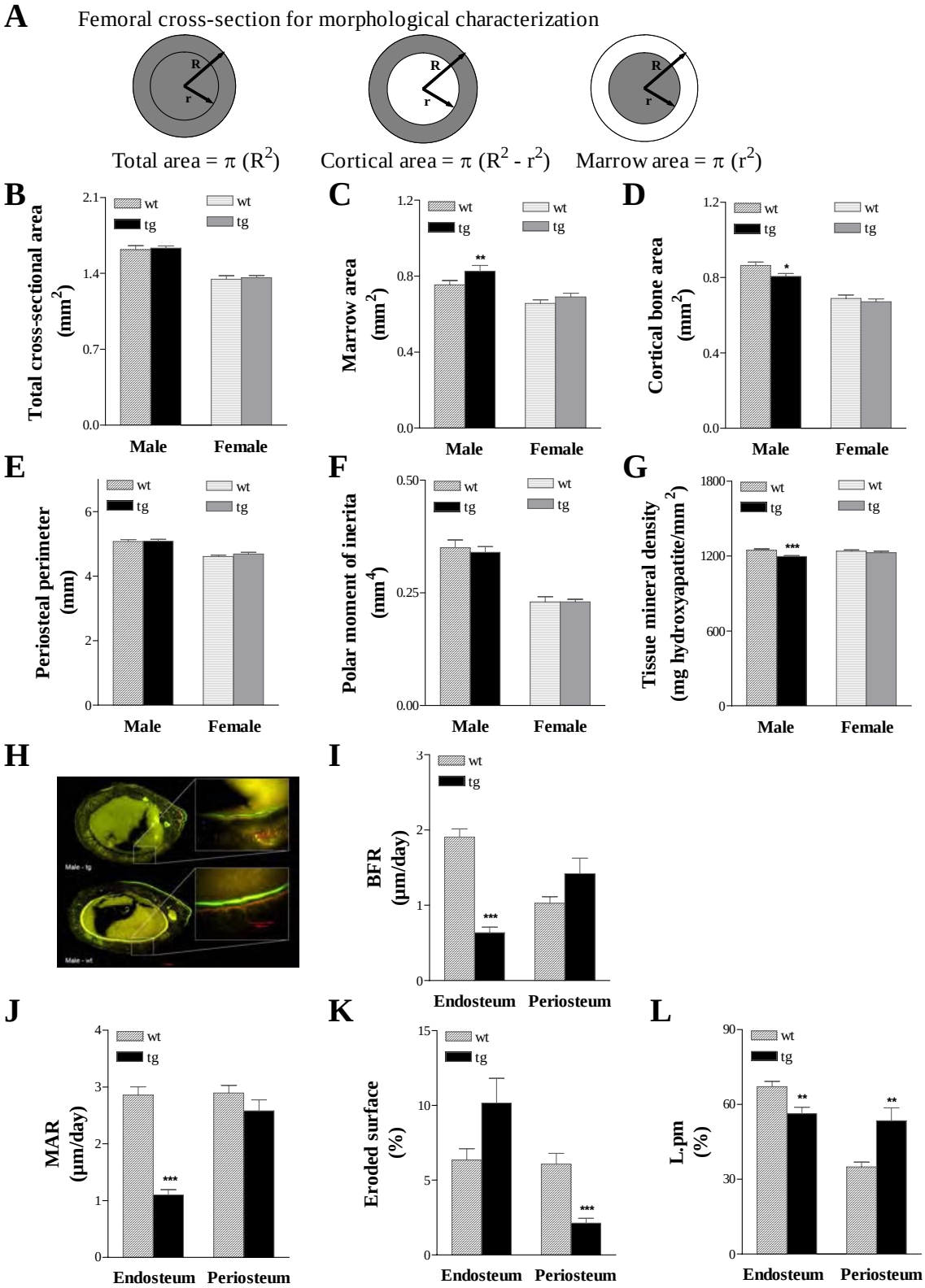

A
Femoral cross-section for morphological characterization
R
r
Total area = π (R2)
Cortical area = π (R2 - r2)
Marrow area = π (r2)
B
Total cross-sectional area (mm2)
C
Marrow area (mm2)
D
Cortical bone area (mm2)
E
Periosteal perimeter (mm)
F
Polar moment of inerita (mm4)
G
Tissue mineral density (mg hydroxyapatite/mm2)
wt
tg
Male
Female
H
I
BFR (µm/day)
Endosteum
Periosteum
J
MAR (µm/day)
K
Eroded surface (%)
L
L.pm (%)

Figure 4

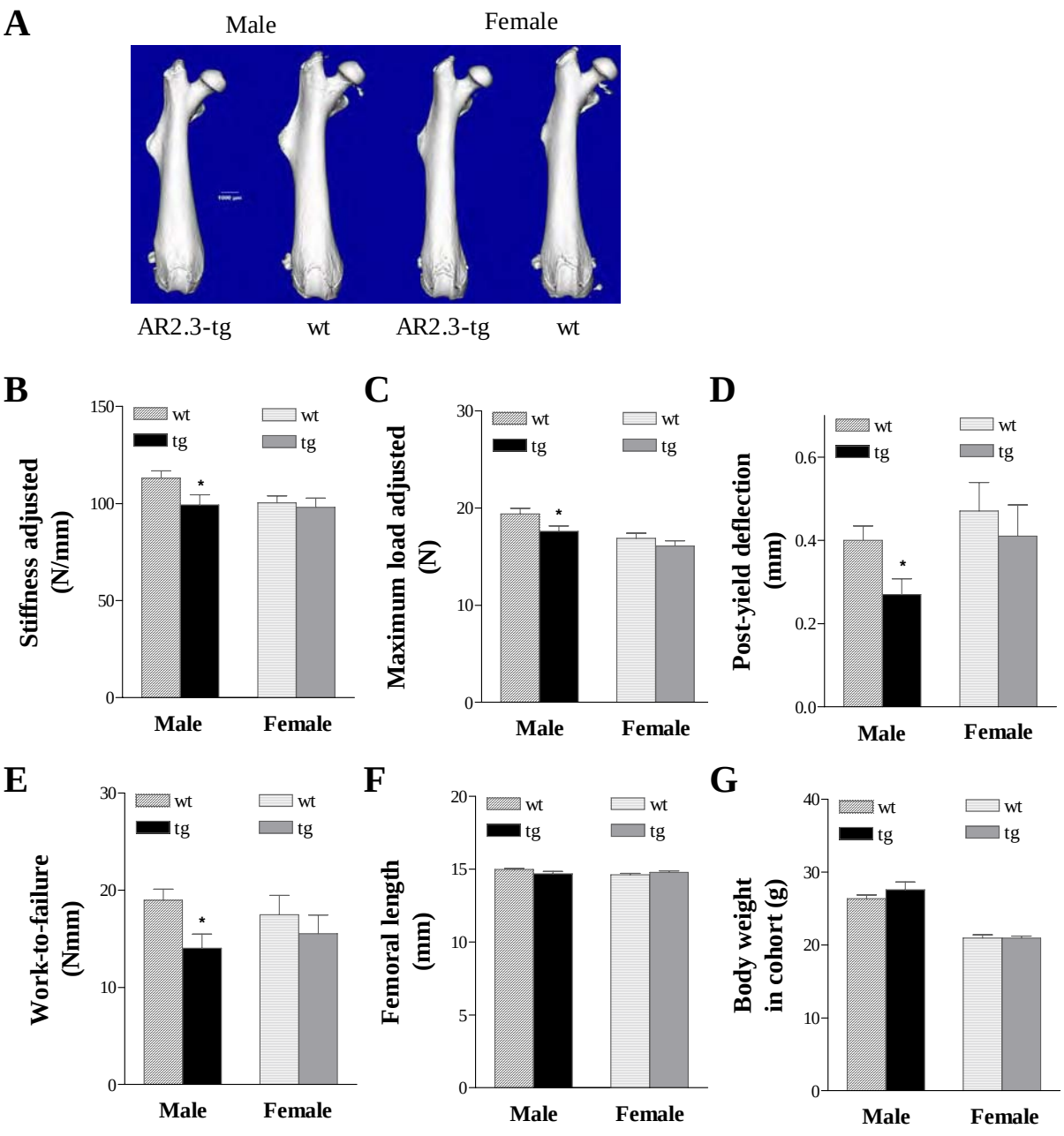

A
Male
Female
AR2.3-tg
wt
AR2.3-tg
wt
B
Stiffness adjusted (N/mm)
C
Maximum load adjusted (N)
D
Post-yield deflection (mm)
E
Work-to-failure (Nmm)
F
Femoral length (mm)
G
Body weight in cohort (g)
wt
tg
Male
Female

Figure 5

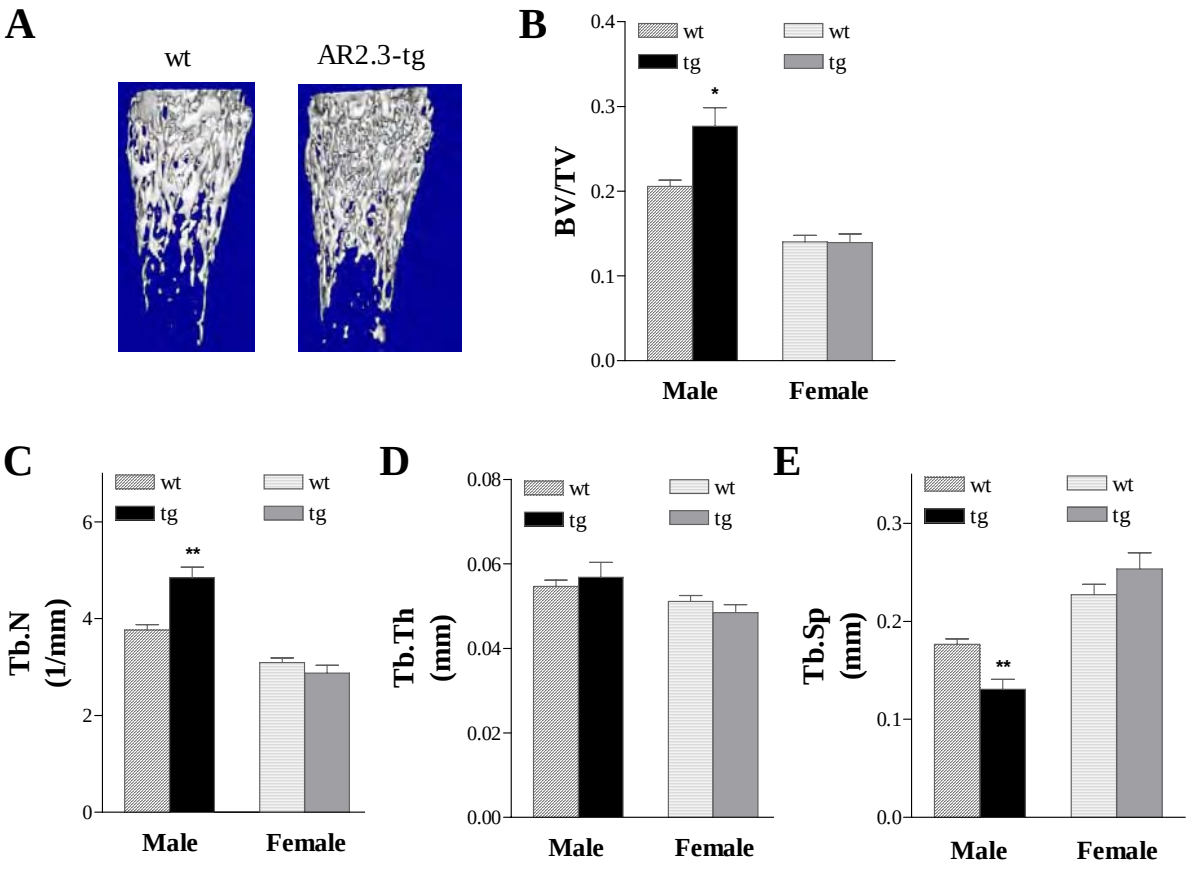

A
wt
AR2.3-tg
B
wt
tg
wt
tg
*
BV/TV
0.4
0.3
0.2
0.1
0.0
Male
Female
C
wt
tg
wt
tg
**
Tb.N (1/mm)
6
4
2
0
Male
Female
D
wt
tg
wt
tg
Tb.Th (mm)
0.08
0.06
0.04
0.02
0.00
Male
Female
E
wt
tg
wt
tg
**
Tb.Sp (mm)
0.3
0.2
0.1
0.0
Male
Female

Figure 6

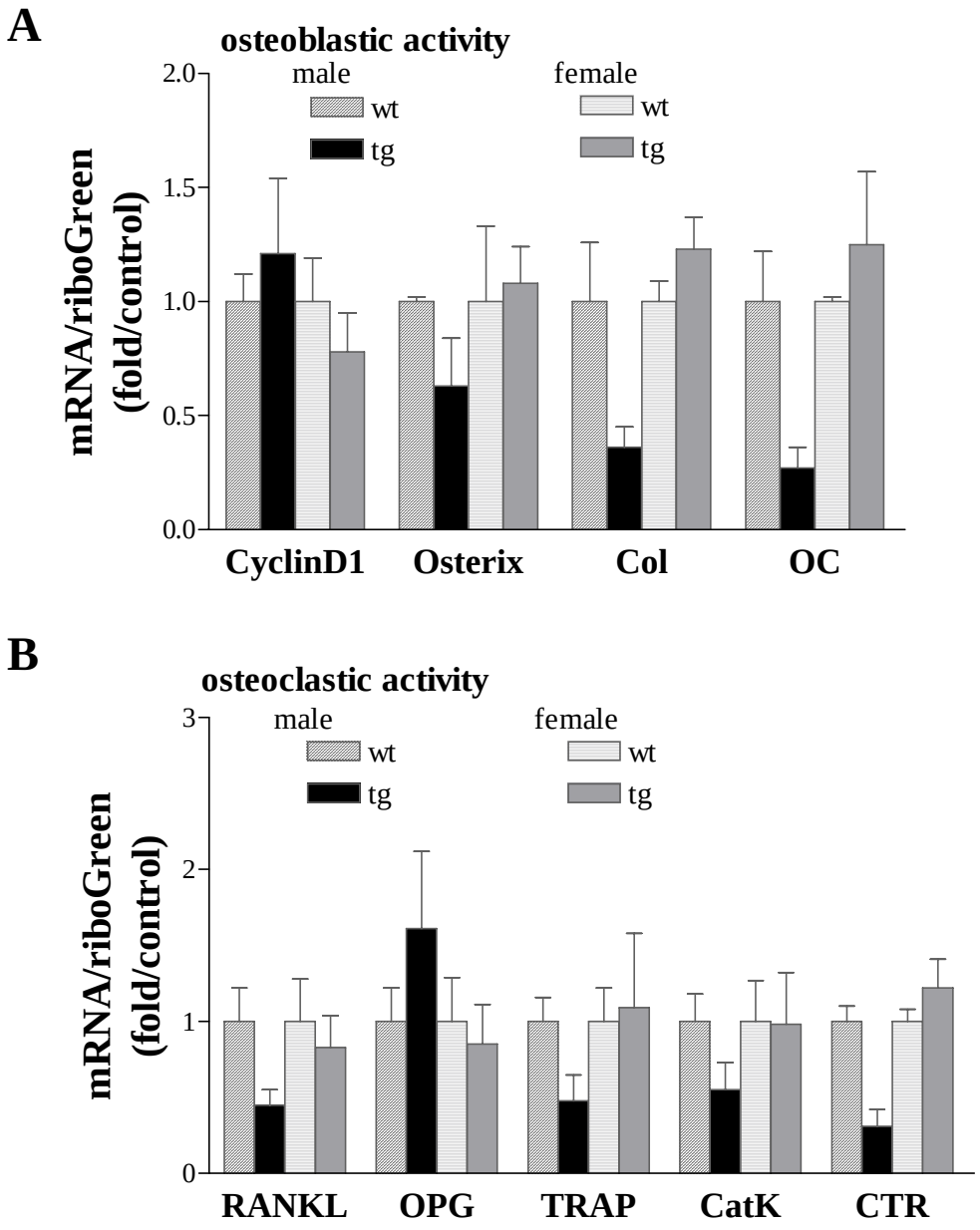
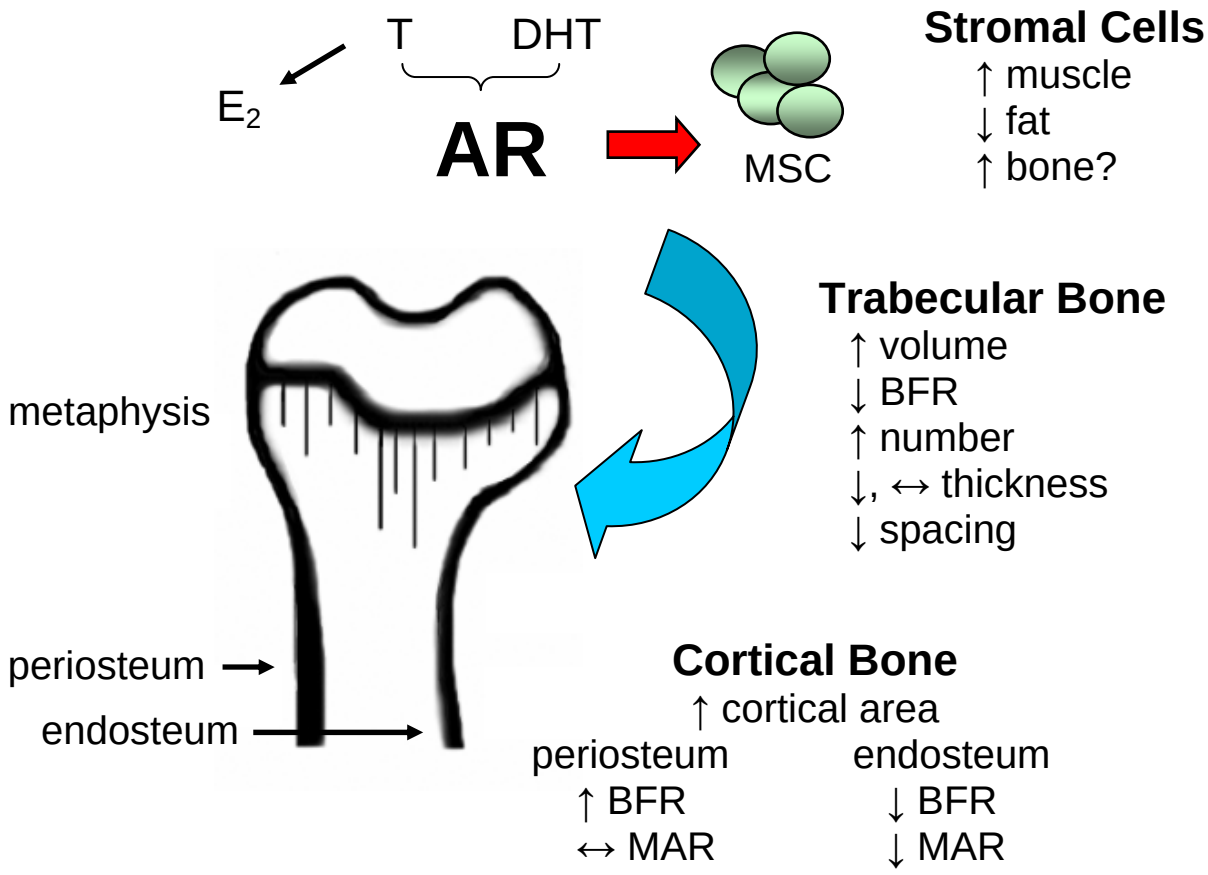

A
osteoblastic activity
male
female
wt
tg
wt
tg
mRNA/riboGreen (fold/control)
2.0
1.5
1.0
0.5
0.0
CyclinD1
Osterix
Col
OC
B
osteoclastic activity
male
female
wt
tg
wt
tg
mRNA/riboGreen (fold/control)
3
2
1
0
RANKL
OPG
TRAP
CatK
CTR

Figure 7


T
DHT
E_2
AR
MSC
Stromal Cells
↑ muscle
↓ fat
↑ bone?
Trabecular Bone
↑ volume
↓ BFR
↑ number
↓, ↔ thickness
↓ spacing
metaphysis
periosteum
endosteum
Cortical Bone
↑ cortical area
periosteum
↑ BFR
↔ MAR
endosteum
↓ BFR
↓ MAR

Appendix 4:

Semirale A, Zhang X-W, Wiren KM: DHT Treatment Reverses Gonadectomy-Induced Changes in Fat and Lean Mass in Male but Not Female Mice. (Abstract#T203) J Bone Miner Res, 2007

DHT Treatment Reverses Gonadectomy-Induced Changes in Fat and Lean Mass in Male but Not Female Mice

A. A. Semirale, X. Zhang, K. M. Wiren. VA Medical Center, Oregon Health & Science Univ, Portland, OR, USA.

Abstract: Androgens have pervasive effects on target tissues including muscle, fat and bone. To characterize the role of androgen receptor (AR) signaling on body composition, an experimental paradigm of protracted hormone ablation followed by steroid replacement was employed. B6D2F2 control mice were sham operated or gonadectomized at 3 months of age. The effect of nonaromatizable dihydrotestosterone (DHT) was determined after an 8 week delay, to provide time for gonadectomy-induced changes in body composition to develop before intervention. The effect of androgen was assessed by dual energy x-ray absorptiometry (DXA) following 6 weeks of treatment with either DHT or placebo pellets. In control males (n = 8-11), lean mass as a % of body weight was significantly reduced (7.7 %, $p < 0.001$) following orchiectomy (ORX) while conversely, % fat mass increased (7.6 %, $p < 0.001$) vs. sham controls. DHT treatment was beneficial, improving or fully restoring body composition changes induced by ORX compared to placebo. In females (n = 4-10) a similar trend was observed with increased fat (2.2 %) and reduced lean mass (2.3 %) after ovariectomy (OVX). Surprisingly, females were resistant to the effects of DHT to restore body composition. To determine whether changes in bone metabolism contribute to body composition differences, transgenic mice with skeletally-targeted AR overexpression in mature osteoblasts (driven by the 2.3 kb fragment of type I α1 collagen promoter) were characterized. AR2.3-tg mice demonstrated changes in body composition after gonadectomy that mirrored wild types (n=3-9). Again similar to wild-type controls, DHT improved ORX-induced alterations in % fat and % lean mass in AR2.3-tg males but not females. In contrast to gonadectomized models, DHT treatment in intact wild type mice had a negative impact on body composition in both males and females. Males (n = 14-23) lost 4.4 % lean mass ($p < 0.05$) and increased fat mass by 4.3 % ($p < 0.05$) compared to placebo, with similar but less dramatic changes observed in females (n = 14-26). These results show both males and females increase fat and lose lean mass following gonadectomy. In the bone-targeted AR-overexpression model, enhanced androgen signaling does not influence the response to DHT suggesting that circulating factors derived from bone likely do not play a role in the body composition response to androgen therapy. Combined, these data indicate that systemic DHT positively influences body composition changes after a prolonged hypogonadal state in males but is ineffective in females over the same time frame. Furthermore, DHT treatment in the intact animal results in detrimental changes in body composition and should be approached with caution.

Appendix 5:

Zhang X-W, Semirale A, **Wiren KM**: Dissection of Androgen Receptor Signaling: Reconsideration of Direct Anabolic Action in Mature Bone. (Abstract#T202) J Bone Miner Res, 2007

Dissection of Androgen Receptor Signaling: Reconsideration of Direct Anabolic Action in Mature Bone

X. Zhang, A. A. Semirale, K. M. Wiren. VA Medical Center, Oregon Health & Science Univ, Portland, OR, USA.

Abstract: The effects of androgen on bone remain poorly characterized and understudied. To develop insight into the cell types important in mediating androgen action, we constructed and compared two distinct transgenic lines employing different α1(I)-collagen promoter fragments to control skeletally-targeted AR overexpression. The col3.6 AR-transgenic (AR3.6-tg) mice demonstrate AR overexpression throughout the osteoblast lineage including the periosteum, while col2.3 (AR2.3-tg) mice have more restricted overexpression in mature osteoblasts and osteocytes. Complex skeletal analysis using morphological characterization by µCT, dynamic and static histomorphometric analysis, dual-energy x-ray absorptiometry (DXA), biomechanical testing and gene expression studies all indicate that androgen signaling in mature osteoblasts during growth produces a low turnover state, and the consequences are detrimental to overall matrix quality and bone strength. To determine the consequences of androgen treatment in the adult and whether nonaromatizble dihydrotestosterone (DHT) may be effective for treatment of post-menopausal bone loss in an animal model, 3 month old male and female B6D2F2 control mice were gonadectomized. Six weeks of treatment with DHT or placebo pellets was delayed until age 5 months to allow rapid bone turnover to stabilize, thus minimizing the impact of anti-resorptive effects of androgen. DXA demonstrated that systemic DHT administration significantly increased BMD and BMC in both sexes, to reverse loss sustained after a prolonged hypogonadal state (n = 14-26). To test the consequences of enhanced androgen signaling targeted to bone in the adult, male and female AR3.6-tg and AR2.3-tg mice were treated in the same manner. In both AR-tg families and in contrast to wild-type mice, DHT replacement did not improve either measure versus placebo pellet (n = 3-14). Taken together, these results indicate that improvements in bone mass with androgen treatment after gonadecomy in wild-type mice are likely mediated through effects on extra-skeletal tissues, not osteoblasts. Consistent with detrimental effects of enhanced androgen signaling on bone quality, DHT treatment in intact controls significantly reduced bone mass in both males and females. These findings demonstrate that after a sustained hypogonadal period, androgen effectively treats bone loss but that enhanced androgen signaling directly in bone is not anabolic in either male or female adults. The data suggests that targeting androgen response to mature osteoblasts is not beneficial for bone formation, and raise concerns regarding androgen administration or anabolic steroid abuse in healthy individuals in both sexes.

Appendix 6:

Semirale A, Wiren KM: Therapeutic Considerations Regarding Androgen Administration. Sun Valley Workshop on Skeletal Tissue Biology, 2007

***Awarded Alice L. Jee Memorial Young Investigator Award**

Therapeutic Considerations Regarding Androgen Administration

A. A. Semirale, K. M. Wiren. VA Medical Center, Oregon Health & Science Univ, Portland, OR, USA.

Androgens are known to have pervasive effects on target tissues including muscle and fat, yet the effects on bone remain poorly characterized. To gain insight into the cell types important for mediating androgen action, we constructed and compared two distinct transgenic lines of mice employing different α_1 (I)-collagen promoter fragments to control skeletally-targeted androgen receptor (AR) overexpression. Histomorphometric and biomechanical analyses revealed compromised bone strength with AR overexpression in bone during development. The role of AR signaling in the adult was characterized *in vivo* using an experimental paradigm of hormone ablation followed by steroid replacement. Control and AR-tg mice were sham operated or gonadectomized at 3 months of age and the effect of nonaromatizable dihydrotestosterone (DHT) was determined after an 8 week delay, allowing for gonadectomy-induced changes to develop. Following 6 weeks of treatment, the effects of androgen on bone and whole body composition was assessed by DXA. In control mice, systemic DHT administration significantly increased BMD and BMC in both sexes, reversing the loss sustained after a prolonged hypogonadal state. In contrast, in AR-tg mice DHT replacement did not improve either measure compared to placebo. DHT treatment was also beneficial to body composition, improving or fully restoring alterations in lean/fat mass after gonadectomy in controls but not AR-tg mice. Further, DHT treatment in intact mice had a negative impact on body composition, reducing lean mass and increasing fat. These findings suggest androgen administration has therapeutic advantages in the hypogonadal, but should be approached with caution in healthy adults.